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Michelle Marie Moore

Louisiana State University and Agricultural & Mechanical College

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**IDENTIFICATION OF ANTIGENS OF *EDWARDSIELLA ICTALURI*, THE CAUSATIVE
AGENT OF ENTERIC SEPTICEMIA OF CATFISH (ESC)**

A Dissertation

**Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy**

in

**The Interdepartmental Program in
Veterinary Medical Sciences through
The Department of Veterinary Microbiology and Parasitology**

by

**Michelle Marie Moore
B.S., Oregon State University, 1986
M.S., Oregon State University, 1991
May 1998**

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DEDICATION

To Grandpa Moore

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ABSTRACT

Edwardsiella ictaluri, causative agent of enteric septicemia of catfish (ESC), is the primary bacterial pathogen of commercially produced channel catfish (*Ictalurus punctatus*). The purpose of this study was to make progress towards development of a successful ESC vaccine by generating and characterizing a pool of *E. ictaluri* antigens and determining if they were protective against ESC in catfish. Antigenic protein expression was evaluated in *E. ictaluri* cells under different conditions of growth using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and western blotting with pooled convalescent catfish serum (CCS). Results showed protein bands separated by one-dimensional PAGE may consist of one or more antigenic and non-antigenic proteins, and strain and culture temperatures do not effect expression of antigenic proteins, but culture media does. A 57 KD antigenic protein was only expressed by *E. ictaluri* grown in minimal media, and antigenic proteins of 58 and 71 KD were expressed at different levels in minimal or rich media. An *E. ictaluri* genomic library in an *Escherichia coli* expression vector was screened using goat anti-*E. ictaluri* serum (GAI) resulting in isolation of 32 clones expressing antigenic *E. ictaluri* proteins. Encoded genes and expressed antigenic proteins of nine clones were analyzed by partial DNA sequencing, 2D-PAGE and western blotting with GAI and CCS. The DNA inserts of three clones were double-strand sequenced. Four putative open reading frames were identified in the insert of clone 4d6 corresponding to antigenic proteins of 63, 20 and 18 KD expressed by both the clone and *E. ictaluri* cells. Genes encoding these proteins had no homology with other known genes. Overlapping inserts of clones 5d2 and 5d3 encoded homologs of *E. coli* partial genes *serA* and *pgk*, and complete genes *rpiA*, *iciA*, *yggE*, *yggB* and *fda*. Cloned antigenic *E. ictaluri* proteins of 33, 27, 35 and 45 KD were putatively identified as products of *yggE*, *rpiA*, *iciA* and *fda* respectively. Protective capabilities of vaccines of cloned antigenic proteins were evaluated in catfish. All vaccine treatments were protective against *E. ictaluri* challenge, however results were inconclusive due high levels of cross-reactive protection of *E. coli*, host strain of the cloning vector.

CHAPTER 1: LITERATURE REVIEW

CATFISH AQUACULTURE

Channel Catfish (*Ictalurus punctatus*)

Channel catfish, *Ictalurus punctatus*, originally native to North America, are found in Mexico, in the United States along the Gulf of Mexico, and within the Mississippi River Valley. They are bottom dwellers preferring a sand, gravel or rubble substrate and their natural habitat is moderate to swift-flowing clear water streams. Due to stocking by state and federal hatcheries, channel catfish can now be found in many fresh and brackish water habitats. Through introduction they have become widely distributed throughout the United States and in many other countries (Tucker and Robinson 1990).

Channel catfish are found in deep protected holes when they are not feeding. Feeding occurs at dusk and dawn with young catfish feeding mostly on aquatic insects in shallow water and adults feeding on aquatic insects, snails, crawfish, fish and plants in deep water. A channel catfish in the wild may take up to four years to reach 1 pound; they are reported to live up to 40 years and may reach more than 50 pounds (Tucker and Robinson 1990).

History of Channel Catfish Aquaculture

Channel catfish are a popular sport fish in the Southeastern and Midwestern United States. Work conducted at state and federal hatcheries prior to 1960 laid the groundwork for production of fry and fingerlings for large scale commercial culture. In the early 1960's channel catfish aquaculture began to occur on a commercial scale in Arkansas (Tucker and Robinson 1990). Currently 15 states are involved in commercial catfish production. The four major catfish producing states are Mississippi, Alabama, Arkansas and Louisiana, which respectively produce 68, 12, 11 and 6% of the foodsize catfish produced in the United States (Brostrom 1997).

Channel catfish were already a popular food fish in the south when catfish farming got started. In other parts of the country, however, catfish were not valued as food fish. National advertising, food quality, and year round availability have succeeded in making catfish the fourth

most frequently consumed fish after tuna, pollack and salmon in the United States (Tucker and Robinson 1990).

Catfish Production

Broodstock

Channel catfish reach sexual maturity at two to five years of age (Tucker and Robinson 1990). Females are at least three pounds and males at the same age are usually larger (Jensen 1990). Mature channel catfish develop secondary sexual characteristics that are most evident at spawning time. The male is darker than the female, with thickened lips and a broader head with muscular pads. Females develop a soft swollen abdomen due to egg development (Jensen 1990; Tucker and Robinson 1990).

Spawning

Spawning occurs when water temperatures are 70-84 °F. Spawning season begins when the minimum water temperature stabilizes at 70 °F and lasts about two months. In southern Louisiana spawning season is from mid-April to June (Jensen 1990). The open pond method is the most commonly used spawning technique. Broodfish are stocked in spawning ponds in late winter or early spring at a male to female sex ration of 50:50 to 40:60. Spawning containers are placed on the pond bottom to provide nesting sites. The male prepares the nest and incubates the spawned egg mass, with an average spawn containing about 25,000 eggs. The adhesive eggs form a gelatinous mass with egg masses weighing about two pounds. Eggs are light yellow when newly spawned and darken with age (Jensen 1990; Tucker and Robinson 1990).

Egg and Larvae Development

At most commercial catfish farms the egg masses are removed from the spawning ponds and transferred to a hatchery (Tucker and Robinson 1990). Eggs are placed in troughs supplied with continuous flowing water, and rotating paddles are used to keep water circulating around the egg masses. The optimal hatchery water temperature is 78-82 °F. Eggs hatch eight days after fertilization in 78 °F water, hatching time is longer in lower water temperatures and shorter when the temperature is higher. Newly hatched larvae are golden colored and school in a tight cluster

at the bottom of the hatchery trough. At this stage the larvae, or sac-fry, get their nourishment from an attached yolk sac. Depending on water temperatures, the yolk sac is absorbed in 3-5 days and the larvae become free swimming. The larvae, or fry, then darken and begin swimming at the surface looking for food. Fry are usually kept in the hatchery troughs and fed a high protein feed for six to ten days, after which they are transferred to earthen outdoor nursery ponds (Jensen 1990; Tucker and Robinson 1990).

By late summer to mid-fall the fry will grow to four to six inches in length. At this size fish are stocked into production ponds for final grow out to market size. Marketable foodfish range from three-fourths to two pounds. In Louisiana the production cycle from spawning to food fish is 14 to 18 months (Jensen 1990; Tucker and Robinson 1990).

Crop Value

The total surface acres used for catfish production in the United States in 1996 was 177,000 acres in 15 states. Total sales for the 15 catfish producing states totaled 417 million dollars in 1996, with 389 million dollars being in foodsize fish. The 15 states produced a total of 506.8 million pounds of foodsize catfish with an average value per pound of \$0.77. Of those totals, Louisiana used 13,700 acres to produce 29.5 million pounds of foodsize catfish valued at 20.9 million dollars (Brostrom 1997).

ENTERIC SEPTICEMIA OF CATFISH

The Causative Agent (*Edwardsiella ictaluri*)

History

Enteric Septicemia of Catfish (ESC) was first described in channel catfish in 1976 and was found to be due to a previously unidentified species of bacteria in the genera *Edwardsiella* (Hawke 1979). The organism was subsequently named *Edwardsiella ictaluri* after the generic name of the host catfish (Hawke, et al. 1981). Since it was first described, *E. ictaluri* has become the biggest disease problem facing the catfish aquaculture industry. From 1985, ESC has run between 22% to 38% of the total case load of fish diagnostic laboratories in the Southeastern United States. The average incidence of ESC from 1991 to 1995 was 28.7% (Mitchell 1997).

The overall economic impact of ESC due to losses and treatment costs is estimated to be 19 million dollars annually (Thune, et al. 1997b).

Morphology and Biochemical Characteristics

Edwardsiella is in the family Enterobacteriaceae, which includes the genera *Escherichia*, *Salmonella*, *Shigella* and *Yersinia*. *Edwardsiella ictaluri* is a Gram negative rod measuring 0.75 x 2.25 µm at 26°C (Hawke 1979), lengthening to 5-7 µm at 37 °C (Plumb and Vinitnantharat 1989). The organism has peritrichous flagella and is weakly motile at 25-30 °C; motility is reduced at 30°C (Hawke, et al. 1981). In brain-heart infusion (BHI) broth and complete defined media (CDM) growth is optimal at a pH of 7.0-7.5 and a temperature of 25-30 °C (Plumb and Vinitnantharat 1989; Collins and Thune 1996). Like other members of the family Enterobacteriaceae, *E. ictaluri* is cytochrome oxidase negative, indole negative, reduces nitrate to nitrite, and does not produce pigment (Hawke, et al. 1981). In BHI the organism is and capable of fermenting glucose, fructose, galactose, mannose and ribose with the production of gas. In triple sugar iron, the reaction is alkaline over acid with no H₂S and little to no gas production (Hawke 1979; Hawke, et al. 1981; Waltman, et al. 1986; Plumb and Vinitnantharat 1989). Collins and Thune (1996) found that in CDM the organism grows well with fructose, galactose, glucose, glycerol, maltose or ribose as a carbon source; growth occurs at a reduced rate with mannose, sucrose or no sugar supplementation (Collins and Thune 1996). The minimal requirements for growth of *E. ictaluri* were shown in a defined minimal media to consist of a basal salt solution, glucose, magnesium sulfate, pantothenic acid, and niacinamide in addition to specific amino acids that depend on the requirements of individual strains (Collins and Thune 1996).

Bacterial Components

Extracellular enzymes and agglutinins. *Edwardsiella ictaluri* does not produce extracellular proteases, lipases, esterases, pectinase, alginase, collagenase, chitinase, or hyaluronidase; however, it does degrade chondroitin sulfate, a major component of cartilage, it has a cell-bound type of hemolysin (Waltman, et al. 1986) and both mannose-resistant and

mannose-sensitive hemagglutinins (Wong, et al. 1989). Virulent strains of *E. ictaluri* have been observed to have a greater ability to degrade chondroitin sulfate than avirulent strains (Stanley, et al. 1994).

Capsule and fimbriae. Wong et al. (1989) found *E. ictaluri* cells lacked fimbriae or a significant glycocalyx on their surface, but did possess unique polyphosphate granules in their cytoplasm which were not found in other *Edwardsiella* species. Stanley et al. (1994) found virulent strains of *E. ictaluri* possessed a glycocalyx but avirulent strains did not.

Flagella. *Edwardsiella ictaluri* cells have peritrichous flagella. Newton and Triche (1993b) used ultra-structural and electrophoretic techniques to examine purified flagella preparations from ten *E. ictaluri* isolates. Their results showed *E. ictaluri* flagella were un-sheathed and made up of two flagellin proteins with apparent molecular weights of 42 and 38 KD. The isolation of two flagellins with different molecular weights was not expected, and the mechanism responsible for the production of two flagellins was not determined, although several possibilities were suggested. One possibility is the bacteria have two different flagella on their surface. A second possibility is that a single flagellum is made up of both of the flagellin proteins. A third possibility is phase variation, with individual bacteria expressing one type of flagellin, while other bacteria in the same population are expressing the other type of flagellin (Newton and Triche 1993b).

Lipopolysaccharide. Like other gram negative bacteria, *E. ictaluri* has an outer membrane consisting of a phospholipid bilayer with lipopolysaccharide (LPS) on the outer surface (Myrvik and Weiser 1988). The LPS of *E. ictaluri* has been examined electrophoretically by a number of researchers and the majority of the strains analyzed have shown the LPS to be of the smooth type (Bertolini, et al. 1990; Newton and Triche 1993a). Bertolini et al. (1990) analyzed 32 isolates of *E. ictaluri* from fish and found only one isolate with LPS which lacked O side chains, they considered this a rough mutant. Weete et al. (1988) Found the LPS of *E. ictaluri* ATCC 33202 to be rough; however, Newton and Triche (1993) found 40 *E. ictaluri* isolates, including ATCC 33202, were of the smooth type. Newton and Triche (1993) suggested

the isolate used by Weete et al. (1988) had mutated to the rough type due to repeated passage on artificial media.

Outer membrane proteins. The LPS of the outer membrane is embedded with lipoprotein and proteins (Myrvik and Weiser 1988). Newton et al. (1990) determined the outer membrane protein (OMP) profiles of 33 *E. ictaluri* isolates by analyzing sodium N-lauroyl sarcosinate OMP extracts by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). They identified ten OMP bands. One major OMP band with an apparent molecular mass of 35 KD made up 60% of the total OMP fraction. The remaining OMP fraction was made up of nine minor bands with apparent molecular masses of 71, 51, 46, 43.5, 38.5, 37.5, 31.5, 28.5 and 19.5 KD. Stanley et al. (1994) observed virulent strains of *E. ictaluri* had more surface proteins than avirulent strains.

Homogeneity

Previous studies on channel catfish isolates of *E. ictaluri* have demonstrated a very high biochemical and serological homology as well as high homology in protein and LPS expression. Waltman and colleagues (1986) examined the biochemical characteristics of 119 isolates of *E. ictaluri* collected from different geological regions over an eight year period. Of the 86 biochemical tests used, the only variations among isolates they found were in production of gas from formate, production of gas from glucose at 37 °C and production of H₂S. Bertolini et al. (1990) demonstrated serologic homogeneity between 32 isolates of *E. ictaluri* by microagglutination assays and SDS-PAGE Western blotting using rabbit antiserum. They showed protein and LPS homology between the same isolates using SDS-PAGE. The homology of outer membrane proteins was demonstrated between 28 *E. ictaluri* isolates using SDS-PAGE (Newton, et al. 1990), and structural homology in the LPS composition was shown in 40 *E. ictaluri* isolates examined by SDS-PAGE (Newton and Triche 1993a). Plumb and Klesius (1988) showed antigenic homogeneity between 14 *E. ictaluri* isolates using catfish antiserum and SDS-PAGE Western blotting.

Given the high homogeneity between *E. ictaluri* strains, it is surprising that Collins and Thune (1996) found the specific amino acids required for growth varied between strains. They suggested the restriction endonucleases that *E. ictaluri* cells possess may act to restrict the intrusion of foreign DNA and could account of the homogeneity of major phenotypic traits and the persistence of minor phenotypic mutations.

The Effects of Culture Conditions on Protein Expression

Other researchers have used denatured two-dimensional PAGE (2D-PAGE) to evaluate differences in protein induction of bacteria under various stress conditions, showing multiple proteins are repressed or induced under different conditions of growth (Kwaik, et al. 1993; Rafie-Kolpin, et al. 1996). The effects of growth in minimal media have been examined in *Brucella abortus* using 2D-PAGE, with results showing induction of 14 new proteins compared to proteins induced by growth in a rich medium (Rafie-Kolpin, et al. 1996).

Protein expression under various culture conditions has not been evaluated in *E. ictaluri*. Previous studies evaluating antigen expression of *E. ictaluri* used enriched culture media such as blood agar, BHI broth, or trypticase soy agar, and different researchers have used different culture temperatures and different bacterial strains (Plumb and Klesius 1988; Baldwin 1997). Antigenic protein expression of *E. ictaluri* has not been evaluated in the defined minimal media (MM19) described by Collins and Thune (1996), or compared between other culture conditions such as differences in temperature.

The Disease Enteric Septicemia of Catfish (ESC)

Distribution and Species Affected

Edwardsiella ictaluri is found throughout the contiguous United States and Thailand. It causes disease primarily in channel catfish in North America, though it has also been isolated from white catfish (*Ictalurus catus*) and brown bullhead (*Ictalurus nebulosus*; Plumb and Sanchez 1983). Natural *E. ictaluri* isolates have been reported from non-ictalurid fish showing signs of disease including green knife fish (*Eigemannia virescens*; Kent and Lyons 1982), danio (*Danio devario*) from Florida (Waltman, et al. 1985), rosy barbs (*Puntius conchonius*) imported into

Australia from Singapore (Humphrey, et al. 1986), walking catfish (*Clarius batrachus*) in Thailand (Kasomchandra, et al. 1987), and sea bass (*Dicentrarchus labrax*) in Spain (Blanch, et al. 1990). Experimental infections have shown that tilapia (*Tilapia aurea*), European catfish (*Siluris glanis*), rainbow trout (*Oncorhynchus mykiss*) and chinook salmon (*O. tshawytscha*) were all susceptible to infection but golden shiners (*Notemigonus crysoleucas*), largemouth bass (*Micropterus salmoides*), bighead carp (*Aristichthys nobilis*), white sturgeon (*Acipenser transmontanus*) and striped bass (*Morone saxatilis*) were not (Plumb and Sanchez 1983; Plumb and Hilge 1987; Baxa, et al. 1990).

Virulence

Edwardsiella ictaluri is a highly virulent organism, with mortalities reaching 60% in some infected catfish populations (Plumb 1984). All sizes of catfish are susceptible to ESC, but production size catfish (over 100 g) are the most severely affected (Plumb 1988). One hundred percent of 2 cm catfish were killed by water borne exposure and intraperitoneal injection of live *E. ictaluri* (Hawke 1979). At 26 °C, 100% of catfish injected with 1.5×10^3 *E. ictaluri* cells were dead within 10 days (Plumb and Sanchez 1983). Catfish immersed in pure *E. ictaluri* broth culture of 4×10^8 colony forming unit per mL for 30 seconds experienced varying mortalities at differing water temperatures, with 97.8% mortality at 25 °C, 46.6% at 20 °C, 25% at 30 °C, 4% at 35 °C and 0% at 15 °C (Baxa, et al. 1992). These results correspond with the seasonality of ESC infections, which occur in the spring and fall when water temperatures are at 22-28 °C (MacMillan 1985).

Transmission and Incidence

Edwardsiella ictaluri was initially thought to be an obligate pathogen because it survived in sterile pond water for only eight days (Hawke 1979). Further studies showed *E. ictaluri* could survive in sterile pond mud for greater than 95 days (Plumb and Quinlan 1986), indicating pond mud may be a reservoir for *E. ictaluri* infections. Transmission of *E. ictaluri* occurs horizontally from fish to fish. Fingerling channel catfish are the most susceptible and infection may result from uptake of the organism from water or mud (Shotts and Plumb 1994). Transmission has also

been shown to occur by cannibalization of *E. ictaluri*-infected fish or by the shedding of *E. ictaluri* from dead fish (Klesius 1994). Carrier fish may serve as a reservoir of infection (Klesius 1992b; Mqolomba and Plumb 1992), and fish-eating birds may act as vectors of transmission (Taylor 1992). The infection may be spread by transfer of infected fingerlings, contaminated nets, or equipment from pond to pond.

Outbreaks of ESC occur seasonally during the spring and fall. Peaks in incidence occur May to June and again September to October. The increased incidents are highly correlated with water temperatures and occur when temperatures reach 22-28 °C (MacMillan 1985). Controlled laboratory experiments have confirmed that the highest mortalities occur when water temperatures are in the 22-28 °C range (Francis-Floyd, et al. 1987; Baxa, et al. 1992).

Clinical Signs

Natural *E. ictaluri* infections may manifest themselves in one of two forms. The first form is an acute gastrointestinal septicemia with rapid mortality. The second is a chronic form involving the olfactory bulb and brain characterized by a "hole in the head" (HIH) lesion which may proceed to septicemia and death (Shotts, et al. 1986).

Clinical signs of ESC vary depending on the water temperature and the size of the infected catfish. The characteristic behaviors of infected fish include reduced feeding activity, listlessness and hanging at the surface vertically with head up and tail down, or erratic swimming at the surface in a spinning motion (Jarboe, et al. 1984; Blazer, et al. 1985; MacMillan 1985; Shotts and Plumb 1994). External signs include petechial hemorrhaging around the mouth, fins and lateral and ventral surfaces; punctate white spots (1-10 mm); swollen abdomen; exophthalmia; pale, swollen gills; and ulceration in the fontanelle of the frontal bones of the skull (MacMillan 1985; Thune, et al. 1993; Shotts and Plumb 1994). Internal signs include anemia; clear yellow or blood tinged ascites fluid; petechial hemorrhaging throughout the visceral mass, peritoneum and musculature; an enlarged, friable liver with macropapular necrotic lesions; an edematous, dark red spleen; and a hypertrophied kidney (Jarboe, et al. 1984; MacMillan 1985; Thune, et al. 1993; Shotts and Plumb 1994). Histological examination of the acute form of the

disease reveals granulomatous enteritis and hepatitis, and granulomatous inflammation with granulation tissue in the skeletal muscles of the jaw. The chronic, HIH form of the disease shows a diffuse granulomatous inflammation of the olfactory bulb and the telencephalon (olfactory lobe) of the brain (Shotts and Plumb 1994). In experimental infections with *E. ictaluri*, catfish develop a moderate normocytic, normochromic anemia and a moderate leukopenia after 96 hours, with hematocrits of $19.53\% \pm 1.9\%$ and WBC $85.8 \pm 4.32 \times 10^3$ cells/mm³ compared to control fish with hematocrits of $25.60 \pm 1.6\%$ and WBC $123.0 \pm 4.30 \times 10^3$ cells/mm³ (Areechon and Plumb 1983; Francis-Floyd 1993).

Early Pathogenesis

The definitive pathogenesis of ESC is unknown. The acute form of ESC is a bacterial septicemia that is thought to occur via the gastrointestinal tract (Shotts, et al. 1986; Newton, et al. 1989). The bacteria appear to enter the gut through ingestion of contaminated food or water and rapidly invade the intestinal lining and disseminate throughout the body (Shotts, et al. 1986). Lesions occur first as infiltrates of macrophages in the lamina propria and submucosa of the anterior intestine. Some of the macrophages in the infiltrates contain bacteria (Newton, et al. 1989).

Baldwin and Newton (Baldwin and Newton 1993) infected catfish with *E. ictaluri* by intra-gastric intubation and followed the early events of pathogenesis. Their results showed *E. ictaluri* cells were able to penetrate the intestinal epithelium. These results were in contrast to those of an earlier, *in vitro* study that had shown *E. ictaluri* cells were unable to penetrate monolayers of cultured epithelial cells (Hep-2 cells, human larynx origin; Janda, et al. 1991b). The reason for the difference in the *in vivo* and *in vitro* studies may be due to the mammalian origin of the cultured cells. In the *in vivo* experiment, the *E. ictaluri* cells penetrated the intestinal epithelium and entered underlying resident macrophages (Baldwin and Newton 1993). This invasion strategy was similar to that of other entero-invasive Enterobacteriaceae (Finlay and Falkow 1989). The *E. ictaluri* cells crossed the intestinal epithelium and were detected in internal organs as early as 15 min post-infection following intra-gastric intubation. Because penetration into the intestinal

epithelium was rapid and caused little damage, it was suggested that *E. ictaluri* used the hosts normal cellular transport systems to cross the epithelium and did not require *de novo* bacterial protein synthesis. Fimbriae were not observed on the *E. ictaluri* cells, and were not thought to play a role in attachment or penetration. Dissemination of *E. ictaluri* cells was thought to occur via the bloodstream within phagocytes (Baldwin and Newton 1993).

The chronic form of the disease, characterized by HH lesions, is thought to occur via olfactory sites (Shotts, et al. 1986; Newton, et al. 1989). The infection appears to start at the olfactory bulbs after bacteria enter the nares in contaminated water or mud, then progresses along the olfactory nerves until it reaches the brain (Shotts, et al. 1986). The olfactory bulbs, olfactory sacs, olfactory nerves, and the anterior olfactory lobes of the brain are commonly surrounded by infiltrates of bacteria or inflammatory cells (Newton, et al. 1989). Involvement in the brain is limited to the telencephalon and meninges. Chronic infection in the brain includes the production of chondroitinase, which may help break down the cartilage in the skull cap, resulting in the HH lesion 2-4 weeks after initial infection (Shotts, et al. 1986).

Morrison and Plumb (1994) followed the early events of pathogenesis in catfish infected with *E. ictaluri* applied directly into the olfactory capsule. The results showed *E. ictaluri* could penetrate the mucosal surface. In contrast to the results of Baldwin and Newton (1993), there was substantial damage to the mucosal layer and distinct fimbriated processes were observed extending from the bacteria. The presence of discrete, *E. ictaluri* containing tunnels within the olfactory epithelium were also observed. Bacteria were phagocytized, but not destroyed by infiltrating leukocytes (Morrison and Plumb 1994). The difference in the results of these two studies (Baldwin and Newton 1993; Morrison and Plumb 1994) suggests there may be two different mechanisms of invasion depending on whether *E. ictaluri* cells are entering via the gastrointestinal tract or through olfactory sites.

A third site of infection has recently been suggested based on uptake of ³⁵S-labeled *E. ictaluri* into channel catfish exposed by bath immersion (Nusbaum and Morrison 1996). This study indicated the gills may be the primary site of entry. This is the only study of the three

(Baldwin and Newton 1993; Morrison and Plumb 1994; Nusbaum and Morrison 1996) that utilized immersion exposure to infect the fish, which is the laboratory exposure method that most closely duplicates conditions of natural exposure. The previous two studies confirmed *E. ictaluri* cells were capable of penetrating the intestinal epithelium and olfactory mucus membranes and disseminating from those sites, but bacteria were administered in high doses directly to these areas (Baldwin and Newton 1993; Morrison and Plumb 1994). The results of the labeling experiment suggest that in a naturally occurring exposure to *E. ictaluri*, the primary site of entry into the catfish may be the gills (Nusbaum and Morrison 1996).

Since *E. ictaluri* infections are correlated with water temperatures, the route of invasion may be effected by water temperatures. The experimental catfish used in the gastric intubation study were held at 24-28 °C (Baldwin and Newton 1993). Fish in the labeling study were held at 21 °C (Nusbaum and Morrison 1996), however, which is outside of the optimal temperature window for *E. ictaluri* infections. The holding temperature of the fish in the olfactory study was not reported (Morrison and Plumb 1994).

Diagnosis

The diagnosis of ESC is based on the observation of the characteristic clinical signs, described previously, and the isolation and identification of *E. ictaluri* as the etiological agent. The organism is isolated from the kidney or HH lesion and inoculated onto tryptic soy agar, McConkey agar, blood agar, or *Edwardsiella* isolation medium (Shotts and Waltman 1990), then incubated at 30 °C for 48-96 hours (Shotts and Plumb 1994).

Presumptive diagnosis is observation of a short, weakly motile or non-motile rod. The organism stains Gram negative, and is cytochrome oxidase negative. There is no indole production in tryptone broth, and the triple sugar iron (TSA) reaction is alkaline over acid. Confirmatory diagnosis is made serologically. A positive identification of *E. ictaluri* can be made using *E. ictaluri* antiserum in slide or microagglutination. Polyclonal or monoclonal antibodies against *E. ictaluri* can be used in fluorescent antibody techniques, or enzyme immunosorbant assays (Rogers 1981; Ainsworth, et al. 1986; Waterstrat, et al. 1989; Klesius, et al. 1991).

Treatment

In antimicrobial susceptibility tests, *E. ictaluri* is sensitive to Terramycin, Furacin, Aureomycin, Streptomycin, Kanamycin and Neomycin, but is resistant to Erythromycin and Sulfamerazine (Hawke 1979). At present, only two antibiotics have approval by the Food and Drug Administration for treatment of ESC, they are oxytetracycline (Terramycin®) and ormetoprim-sulfadimethoxine (Romet®). These antibiotics are administered as medicated feeds. Terramycin is available in a sinking pellet and Romet is available in a floating pellet (Thune 1993). The effectiveness of the antibiotics depends on early treatment since ESC can cause fish to stop eating. In addition, increases in the number of antibiotic-resistant strains of *E. ictaluri* are reducing the effectiveness of these drugs (Thune and Johnson 1992).

THE IMMUNE SYSTEM OF FISH

Relationships and Diversity of Fish

Fish are thought to have evolved from aquatic vertebrates during the Silurian period ('Age of Fishes') of the Paleozoic era 405-425 million years ago (Wallace, et al. 1981). Fish are a diverse group of animals comprised of approximately 21,700 species, which makes up almost half of the known vertebrate species (Fange 1994). There are eight classes in the subphylum Vertebrata, four are fishes. The four fish classes include Placodermi, Agnatha, Chondrichthyes and Osteichthyes. The Placodermi, now extinct, were the first jawless fishes. The Agnatha are jawless fishes represented today by the cyclostomes (lampreys and hagfish). The Chondrichthyes are the cartilaginous fish and include elasmobranchs (sharks, rays and skates) and holocephalans (chimeras). The Osteichthyes are the bony fishes, the largest and most diverse class, with over 20,000 species broken up into two major groups. The first group is the lobe-finned fishes which includes the dipnoans (lungfish) and the crossopterygians (coelacanths). The lobe-finned fishes are thought to have given rise to terrestrial vertebrates. The second, and much larger group is the ray-finned fishes which includes the chondrosteans (sturgeons, paddlefish and bichirs), the holosteans (bowfin and gars) and teleosts (modern bony fish; Bond 1979; Wallace, et al. 1981; Fange 1994).

Comparative immunologists study the immune system of fish because, as the most primitive vertebrates, fish are an important link between invertebrates and higher vertebrates (Kennedy-Stoskopf 1993). The immune system of fish is studied by fish disease researchers investigating how fish respond to pathogens. Phylogenetic diversity is greater in fish than it is in mammals and, although knowledge of the fish immune system is accumulating, the diversity and broad phylogenetic relatedness between fishes makes it impossible to study more than a few representative species. Drawing conclusions from this limited data base has risks, but is unavoidable due to the fact that there are fewer immunologists working on all of the fish species than there are working on one murine species (Kaattari 1992). With the risks in mind, models of the fish immune system have been developed by drawing on examples taken from a number of different species. These models form the framework of the current state of knowledge of fish immunology.

Lymphomyeloid Tissue

In higher vertebrates the primary sources of lymphomyeloid tissue include the lymph nodes (lymphoid), bone marrow (myeloid), thymus and spleen. All fish, with the exception of the cyclostomes, possess a well developed thymus and spleen. Cyclostomes do not have a true thymus and only a rudimentary spleen (Kennedy-Stoskopf 1993). Fish lack lymph nodes and bone marrow, but possess lymphomyeloid tissues that perform similar functions.

Lymphomyeloid tissues are found in different locations in fish depending on their phylogenetic group. Lymphomyeloid tissues are found in the anterior part of the kidney (the head-kidney or pronephros) in teleosts; in the spiral valve of cartilaginous fishes; in the liver, leydig organ (in the esophagus) and epigonal organ (in the gonads) of elasmobranchs; in the cavities of the cartilaginous skeleton in holocephalans; in the head-kidney, meninges and pericardium in chondrosteans; and in the liver of cyclostomes (Kennedy-Stoskopf 1993; Fänge 1994).

Thymus

In most vertebrates, including fish, the thymus is a gland that originates from the pharyngeal pouches and is the first lymphoid organ to develop. The first appearance of lymphocytes occurs 22 days pre-hatch in Atlantic salmon (*Salmo salar*); 3-4 days post-hatch in rainbow trout, rosy barb and carp (*Cyprinus carpio*); and 6-8 days late pre-larval in tilapia (*Sarotherodon mossambicus*; Manning, et al. 1982). In fish, the thymus is a well developed, paired organ present on the dorsolateral region of the gill chamber (Chilmonczyk, 1992; Kennedy-Stoskopf, 1993). All fish have a thymus, with the exception of cyclostomes. The thymus gland consists of lymphocytes supported by a framework of reticulo-epithelial cells surrounded by an epithelial capsule. The largest cellular component of the thymus are lymphocytes. The cortex and outer zones are highly lymphoid, the medulla and inner zones consist of connective tissue with lymphocytes scattered within (Chilmonczyk 1992).

Anterior kidney

The kidneys of most fishes are encapsulated, dark red, slender, elongate organs found running ventrally along the vertebral column. The kidneys are paired, but often in such close proximity to each other that there is fusion along the midline (Bond 1979). The posterior portions of the kidneys are responsible for excretory functions. The anterior portions of the kidneys are the primary hematopoietic organs of teleost fishes. The first appearance of lymphocytes occurs 14 days pre-hatch in Atlantic salmon; 4-6 days post-hatch in rainbow trout, rosy barb and carp; and 13-16 days mid post-larval in tilapia (Manning, et al. 1982). The hematopoietic parenchyma of the anterior kidney is lymphoreticular, with reticular and fixed phagocytic reticular cells present, as well as sinusoids and thin-walled arteries. Based on these properties, the anterior kidney of teleost fish is structurally more analogous to mammalian bone marrow than lymph nodes (Zapata 1979).

Spleen

The spleen is a dark red, triangular organ that lies adjacent to the stomach, to which it is attached by a ligament (Bond 1979). In teleost fish, the spleen is the primary organ where

erythrocytes, neutrophils and granulocytes mature, are processed or stored (Anderson 1974). It is also involved in hematopoiesis, the clearance of macromolecules, antigen degradation and processing, and antibody production (Dalmo, et al. 1997). The first appearance of lymphocytes occurs 42 days post-hatch in Atlantic salmon; 6-8 days post-hatch in rainbow trout, rosy barb and carp; and 30-80 days in juvenile tilapia (Manning, et al. 1982). The spleen of fish contains red pulp and white pulp, as in mammals, but it is relatively undifferentiated and lacks germinal centers (Zapata 1982; Kennedy-Stoskopf 1993). The red pulp contains mostly erythrocytes and a few lymphocytes. The white pulp consists of lymphoid tissue in a reticular network found mainly around blood vessels (Anderson 1974; Zapata 1982). The splenic lymphoid tissue is poorly developed and splenectomized fish show no alteration in immune functions (Zapata 1982).

Gut Associated Lymphoid Tissue (GALT) and the Mucosal Immune System

Lymphoid cells can be found in the lamina propria and intestinal epithelium of all vertebrates including fish. All fish studied, with the exception of the Agnatha, have well organized gut-associated lymphoid aggregates. The aggregates are un-encapsulated and consist of lymphocytes, macrophages, granulocytes and plasma cells. Histological evidence has shown teleosts and some elasmobranchs have a common mucosal immune system, with lymphoid aggregates associated not only with the gut, but also the reproductive tract, skin and gills (Zapata, et al. 1996).

Lymph

Fish lack lymph nodes, but do have lymph or lymph-like fluid. The lymph occurs in spaces around skeletal muscles and connective tissues. The fluid, formed by filtration from blood vessels, contains many leucocytes and a few erythrocytes. In teleosts, the volume of lymph may exceed the volume of blood (Fänge 1994).

Macrophage Aggregates

Macrophage aggregates, or melano-macrophage centers, occur in fish and other poikilothermic vertebrates. They are most commonly found in the spleen, kidney and liver, but may be found in other organs in association with inflammation (Wolke 1992). Macrophage

aggregates consist of focal accumulations of macrophages that usually contain hemosiderin, lipofuscin, ceroid, and melanin (Wolke 1992; Kennedy-Stoskopf 1993). The aggregates are thought to have several functions including humoral and inflammatory immune responses, storage and destruction of endogenous and exogenous agents, and iron recycling (Wolke 1992). Macrophage aggregates may be primitive forms of the germinal centers found in the spleens and lymph nodes of higher vertebrates (Kennedy-Stoskopf 1993).

Non-Specific Immune Responses

Distinction Between Innate Resistance and Adaptive Immunity

Innate resistance is a result of the non-specific, intrinsic mechanisms that act to keep a host free of disease. These nonspecific defense mechanisms include a number of natural protective responses that prevent infection, limit the spread of infection, and remove living and non-living tissue-damaging agents. Adaptive immunity is a specific immune response to a particular antigen that requires a previous exposure to the antigen. With adaptive immunity the response against an antigen is faster and stronger with subsequent exposures, this increase in response is termed memory. Innate resistance does not require any previous exposure to an antigen, and it is not induced by a specific antigen. In addition, nonspecific responses lack memory. That is, they remain constant with repeated exposures to pathogenic agents. Non-specific mediators of innate resistance include physical barriers as well as humoral and cellular factors.

Physical Barriers

Skin. The skin provides a physical barrier to invading microorganisms. The skin of higher vertebrates is composed of non-living, keratinized cells. In contrast, the skin of teleost fish is composed of non-keratinized, living cells (Roberts and Bullock 1980). Wound healing is much more rapid in fish, and the healing processes are different than in mammals (Bullock, et al. 1978). In mammals, a protective scab forms over the wound and hardens in air. Mitotic proliferation of the underlying germinal layer results in the scab being undergrown by epithelial cells until finally it is cast away along with any associated microorganisms. In teleost fish, an

intact barrier is necessary for maintaining osmolarity, and wounds are quickly covered by epithelial Malpighian cells which migrate from the periphery of the wound over the wound surface. Unlike the mammalian scab, which traps and excludes microorganisms, the rapid overgrowing of epithelial cells over a teleost wound occurs at the risk of enclosing microorganisms (Ellis 1981).

Mucus. An additional defense that is found on fish, but absent on most other vertebrates, is an external barrier of mucus. Mucus, the outermost line of defense in fish, inhibits colonization of microorganisms and parasites on the skin as well as on the gills and gastrointestinal mucosa. Goblet cells in these surfaces secrete a continual supply of mucus, which is easily swept away carrying entrapped debris and microorganisms. The major component of mucus is mucin, which is composed mostly of glycoproteins. The rate of mucus secretion increases in response to infection or physical or chemical irritants (Anderson 1974; Ellis 1981; Alexander and Ingram 1992; Kennedy-Stoskopf 1993). In addition to its role as a physical barrier, mucus contains factors that inhibit the growth and establishment of parasites and microorganisms. These factors are discussed below.

Nonspecific Soluble Factors of the Serum and Mucus

Transferrin. Transferrins are non-heme, globular, iron-binding glycoproteins which are found in the sera of all vertebrates. All organisms, including microorganisms and parasites, require iron for metabolisms and growth. Transferrins reduce infections by binding iron, making it unavailable to invading organisms. The low availability of iron restricts bacterial or fungal multiplication until the immune system can respond. In mammals, transferrin is a single polypeptide chain of about 80 KD. In fish, the transferrins have all been found to have molecular weights of 70 to 80 KD (Ingram 1980; Alexander and Ingram 1992). Three genotypes of transferrin in coho salmon (*Oncorhynchus kisutch*) were correlated with degrees of resistance to bacterial kidney disease (BKD; *Renibacterium salmoninarum*). The authors suggested certain genotypes were better at competing for iron than others; though they acknowledged resistance

to BKD may be correlated to transferrin genotype, not directly related to the transferrin protein (Suzumoto, et al. 1977).

Interferon (IFN). Interferons are glycoproteins produced in response to viral infections, immune stimulus and a variety of chemical stimulators. Interferon inhibits virus replication by interfering with viral RNA and protein synthesis. The anti-viral activities of IFN are non-specific (Tizard 1992). In most studies in fish IFN is species specific but not virus specific, as in mammals (Secombes 1994).

In mammals, there are three classes of IFN divided into two types. Type I includes IFN- α and IFN- β , type II is IFN- γ (Alexander and Ingram 1992; Tizard 1992). IFN- α is produced mainly by virus infected monocytes and lymphocytes, IFN- β is produced mainly by virus infected fibroblasts, and IFN- γ is produced by T cells and natural killer cells after exposure to interleukin-2 (IL-2; Tizard 1992). Because IFN- γ is a macrophage activating factor (MAF), it is considered a lymphokine (cytokine). Interferon type I production has not been confirmed in cyclostomes or cartilaginous fish, but it has been well documented in bony fish (Alexander and Ingram 1992; Yano 1996). IFN type I in rainbow trout was shown to provide nonspecific antiviral protection that could be passively transferred to other trout (de Kinkelin, et al. 1982), and a macrophage activation factor with activities similar to IFN- γ has been demonstrated in rainbow trout (Graham and Secombes 1988; Alexander and Ingram 1992).

The putative sequence of IFN has been obtained from the Japanese flatfish, *Paralichthys olivaceus* (Tamai, et al. 1993). The researchers made a cDNA library from RNA extracted from oncogene-immortalized flatfish lymphocytes that had an IFN-like activity. The library was screened for biological activity and a clone expressing an IFN-like protein was sequenced. The cloned protein encoded 138 amino acid polypeptide with a molecular weight of 16 KD (Tamai, et al. 1994). The amino acid sequence had less than 20% homology with mammalian IFN, leading other researchers to suggest the gene product is not an IFN protein (Secombes, et al. 1996).

Enzyme inhibitors. The purpose of enzyme inhibitors is to destroy or inactivate cellular enzymes that have been released by dead or dying host cells. In addition, many invading organisms utilize proteases to damage the host in order to penetrate host cells or obtain essential nutrients (Alexander and Ingram 1992). The serum of mammals contains specific inhibitors for three of the major protease groups, including serine proteinase inhibitors, cysteine proteinase inhibitors and metalloproteinase inhibitors. In addition, mammalian serum also contains α 2-macroglobulin (α 2-M), a nonspecific protease inhibitor capable of inhibiting the enzymes from all of the groups of proteases. All groups of vertebrates have protease inhibitors analogous to α 2-M. Thus far, the α -proteinases analogous to mammalian α 2-M are the only group of protease inhibitors identified in fish (Alexander and Ingram 1992).

The α -antiprotease found in normal rainbow trout serum was shown to neutralize the proteolytic activity of exotoxin from the fish pathogen *Aeromonas salmonicida* in both *in vitro* and *in vivo* studies (Ellis 1981). The protozoan *Cryptobia salmositica* parasitizes both rainbow trout and brook charr (*Salvelinus fontinalis*) but does not cause clinical signs of cryptobiosis (e.g. anemia) in the brook charr. The parasite secretes a metalloprotease that is capable of lysing fish erythrocytes and appears to be associated with virulence. The α 2-M from the blood of rainbow trout and brook charr was shown to neutralize the metalloprotease activity of the protozoan parasite *in vitro* and *in vivo*. Since brook charr were found to have a much higher activity levels of α 2-M than rainbow trout, it was suggested that the α 2-M played an important role in defense against cryptobiosis (Zuo and Woo 1997).

Lysozyme. Lysozyme, also known as muramidase or N-acetylmuramide, is a mucolytic enzyme produced by leucocytes. The specific substrate of lysozyme is β 1-4 linked N-acetylmuramine and N-acetylglucosamine, which form the peptidoglycan layer of the cell walls of bacteria. Gram positive bacteria can be directly damaged by lysozyme, but Gram negative bacteria are not susceptible to lysozyme unless the outer cell wall has been disrupted by complement or enzymes, exposing the inner peptidoglycan layer. When the peptidoglycan cell wall layer is lysed by lysozyme the plasma membrane usually ruptures due to osmotic stress and

the cell dies. The enzyme has antibiotic properties and is widely distributed in nature (Lindsay 1986; Alexander and Ingram 1992).

Lysozyme has been found in the serum, mucus and some tissues of fish (Fletcher and White 1976; Fletcher, et al. 1977; Ourth 1980). In rainbow trout and plaice (*Pleuronectes platessa*) lysozyme has a distribution compatible with a defense function, and is found in tissues rich in leucocytes and at sites where the risk of bacterial invasion is high, such as the mucus and gills (Murray and Fletcher 1976; Lindsay 1986). The lysozymes isolated from fish have molecular weights ranging from 12.5 to 15 KD (Alexander and Ingram 1992).

Grinde et al (1988) compared lysozyme activity between 12 species of fish and found both species and individual variation. The highest lysozyme activities were found in rainbow trout, sea char (*Salvelinus alpinus*), turbot (*Psetta maxima*), halibut (*Hippoglossus hippoglossus*) and catfish (*Anarrhichas lupus*). Rainbow trout had the highest lysozyme activities, about 500 times that of ling (*Molva molva*) and tusk (*Brosme brosme*), the species with the lowest activities.

Proteinases. Proteinases with trypsin-like activity have been identified in the skin mucus and mucus-secreting cell layers of rainbow trout and Atlantic salmon (Hjelmeland, et al. 1983; Braun, et al. 1990). Immunohistochemical examination showed trypsin was present in Atlantic salmon mucus-secreting cells in the epithelium, gills, intestine and epidermis of dorsal skin (Braun, et al. 1990). Because the trypsin-positive tissues comprise most of the external surface of fish, and a common feature of these tissues is mucus secretion, the authors felt that their findings supported the hypothesis that trypsin has a role in the defense systems of fish to environmental pathogens (Hjelmeland, et al. 1983; Braun, et al. 1990).

Agglutinins and precipitins. Agglutination and precipitation both involve the cross-linking of molecules. Agglutination is the aggregation of cells due to cross-linking of molecules attached to the cell surface and precipitation is the aggregation of molecules in solution. If the molecule is large enough, agglutination and precipitation can be brought about by the same substance (Alexander 1985; Alexander and Ingram 1992).

Though they are sometimes referred to as 'natural antibodies', the agglutinins in fish are nonspecific factors that occur in the absence of any specific antigenic stimulation. The agglutinins have functional homology to immunoglobulins, but they are not immunoglobulin molecules. In fish, agglutinins are found in serum, skin mucus and eggs (Fletcher 1982; Ingram 1985; Suzuki 1985; Alexander and Ingram 1992). All of the agglutinins react with monosaccharides found in carbohydrates and glycoproteins. Non-immunoglobulins that react with carbohydrate moieties are generally referred to as lectins (Alexander and Ingram 1992). Natural agglutinins to heterologous erythrocytes have been widely detected in freshwater and marine teleosts and in elasmobranchs (Ingram 1980). Many of the studies showed natural agglutinins with hemagglutinin activity against human blood group determinants as well as a variety of other vertebrate species (Ingram 1985; Suzuki 1985). The role of agglutinins in fish is speculative, but they are thought to have a putative defense function. They may represent primitive immune surveillance molecules. Evidence suggests they are involved in recognition rather than as effectors of pathogen destruction (Fletcher 1982; Alexander and Ingram 1992).

The best studied precipitins in fish are C-reactive protein (CRP) and α -precipitins. Mammalian CRP is in the acute phase protein group, the concentrations of which increase rapidly following tissue damage, inflammation or infection (Alexander and Ingram 1992). In the presence of Ca^{2+} ions, CRP precipitates the so called c-polysaccharides, or phosphocholine containing molecules found widely distributed in nature. Following reaction to its substrate, CRP can activate complement via the classical pathway. It is also an opsonin and can precipitate or agglutinate macromolecules and microorganisms with surface phosphorylcholine (Alexander and Ingram 1992).

The CRP of fish differs from that of mammals in that it is not an acute phase protein, but rather appears to be a normal constituent of fish serum (Ellis 1981). Cross-reactive protein has been found in the serum, mucus and eggs in a number of teleosts and elasmobranchs (Fletcher, et al. 1977; Ramos and Smith 1978; Alexander and Ingram 1992). The CRP of fish have been shown to react with C-polysaccharides of bacteria, some fungi and extracts of a nematode worm

(Baldo and Fletcher 1973). The CRP from rainbow trout has been isolated and characterized as a 66 KD protein made up of two subunits (Murai, et al. 1990)

Another type of precipitin found in the sera of all vertebrates, including fish, are the α -precipitins. These proteins have α -globulin mobilities in immunoelectrophoretic gels (Ellis 1985), and exist in two forms with molecular weights of 170 and 500 KD. α -Precipitin is not a single protein, but rather a group of molecules with different specificities (Alexander 1985). The α -precipitins of fish have been shown to react with extracts of fungi and bacteria and with amylose and amylopectin (Alexander 1985). The α -precipitins are not Ca^{2+} -dependent, and are therefore not CRP (Alexander and Ingram 1992). Observations by Ellis (1985) have led him to suggest the precipitation reactions caused by α -precipitins are due to enzyme-substrate reactions with the formation of insoluble products rather than precipitation of a complex in the manner of true precipitins. The role of α -precipitin in fish is unknown, but Davies and Lawson (1985) speculated it may function as a recognition molecule detecting carbohydrate moieties and, like CRP, it may be a nonspecific defense mechanism.

Complement. Complement is a group of enzymes that react in a regulated cascade in a reaction culminating in disruption of cell membranes. In addition to the lysis of cell membranes, the activation of complement can result in cell-mediated chemotaxis of granulocytes and phagocytic cells, complement-mediated opsonization and complement-mediated inflammation. In mammals, at least 19 proteins make up the complement system. They are all serum proteins, and together they account for about 10% of the serum globulin fraction (Tizard 1992). The main components of mammalian complement are numbered C1 to C9.

There are two convergent pathways of complement activation, the classic pathway and the alternative pathway. These pathways are activated by different mechanisms, but have the same end result. In the classic pathway, antigen-antibody complexes of IgG or IgM start the cascade reaction by activation of the first component, C1. The activation of C1 causes the activation of C4, C2 and C3 leading to the final cascade of C5-C9. The activation of C3 can also occur by the alternate pathway in the absence of antigen-antibody complexes. The alternate pathway is triggered by the activation of properdin by a number of substances, including bacterial

LPS, fungal polysaccharides, plant factors and animal venom. Activated properdin acts on C3 and results in the activation of the complement cascade sequence from C5 to C9, as in the classical pathway activation. The classical pathway requires both Ca^{2+} and Mg^{2+} ions, while the alternate pathway requires only Mg^{2+} (Sakai 1992; Tizard 1992).

Activation of the classic pathway is a specific immune response because it is mediated by antibodies. Activation of the alternate pathway does not require specific antibodies and is a nonspecific immune response. The classic pathway can, however, be activated non-specifically, in the absence of antibody, through direct activation of C1 by the surface proteins of some viruses, by bacteria such as *E. coli* and *Klebsiella pneumonia*, by CRP, or by myelin (Tizard 1992).

Complement can be found in the both the serum and mucus of fish (Harrell, et al. 1976; Kennedy-Stoskopf 1993). Most fish, with the exception of Agnatha, have a complement lytic system analogous to mammals, which can be activated by both the alternate and classic pathways (Harrell, et al. 1976; Giclas, et al. 1981; Ourth and Wilson 1982; Kennedy-Stoskopf 1993). Hagfish appear to only have the terminal components of complement, and these act in a series of aggregations leading to the formation of a membrane-damaging complex, rather than as a true cascade of enzyme reactions (Kennedy-Stoskopf 1993). Components analogous to mammalian C3 and C5 have been identified in rainbow trout, C1 to C4 have been identified in carp, and C1 to C4 and C9 have been identified in elasmobranchs. Properdin has also been observed in fish (Ingram 1980). The full cascade of components equivalent to C1 through C9 and factors B and D have recently been identified in a single fish species, the carp (Yano 1996).

Fish complement has not been completely characterized, but it does appear to have some characteristics different from mammalian complement. Fish complement is more heat labile than mammalian complement, making it more difficult to preserve. It has a lower optimal temperature, higher antimicrobial actions and is more species-specific or species-group specific than mammalian complement (Sakai 1992). Fish complement exhibits lytic activity by activation of the alternative or classical pathways. The lytic activity includes hemolysis, bactericidal and bacteriolytic activities, cytotoxicity, viral inactivation, and detoxification. Fish complement also

has opsonic action thought to be due to a mammalian C3b-equivalent fragment, though a C3b receptor has yet to be identified on fish leukocytes (Sakai 1992).

Nonspecific Cellular Factors

Phagocytes. Phagocytosis involves the chemotaxis, adherence, ingestion and destruction of foreign material by phagocytic cells. Neutrophil granulocytes and mononuclear phagocytes are the primary phagocytic cells in fish, though eosinophils and thrombocytes may also be phagocytic in some fish species (Ainsworth 1992). Neutrophils mainly phagocytize bacteria while macrophages phagocytize any foreign particles, as well as damaged host cells. Fish phagocytes are morphologically and functionally similar to their mammalian counterparts and are a primary defense mechanism against invading organisms. The stimulation and response of phagocytes is nonspecific, but they affect and are effected by products and cells of the specific immune system (Ellis 1981).

In mammals, granular leukocytes, which include neutrophils, eosinophils and basophils, are characterized by their polymorphonuclear nucleus, as opposed to the single rounded nucleus of mononuclear monocytes and macrophages. Neutrophils, also called polymorphonuclear (PMN) phagocytic cells, are cells that respond rapidly, but are incapable of a sustained effort. Monocytes and macrophages are cells that respond more slowly, but are capable of repeated phagocytosis. Neutrophils reach and attack foreign material first, and in dying they attract macrophages to the site of invasion (Tizard 1992). In mammals, granulocytes and monocytes are derived from the same myeloid stem cell in the bone marrow. In fish, which lack bone marrow, phagocyte development occurs in the lymphomyeloid organs. Mature neutrophils are found in blood, peritoneal exudates and tissues (Ainsworth 1992). They are also frequently seen in inflammatory lesions (Ellis 1981). The neutrophil nucleus of bony fish is generally round or slightly oval, rather than PMN. Rainbow trout are unusual in that they do have PMN neutrophils. It was originally thought that fish neutrophils were not actively phagocytic (Ellis 1981). It is now well established that piscine neutrophils are phagocytic, but they are selective for the types of materials they will phagocytize (Finco-Kent and Thune 1987; Ainsworth 1992).

The mononuclear phagocytes include monocytes and macrophages. Monocytes are found in the peripheral circulation, and macrophages are located in the tissues. Although capable of active phagocytosis, monocytes found in the blood are considered immature macrophages (Tizard 1992). In teleosts, macrophages are found widely distributed in tissues including the gills and peritoneum, but are mainly found in the lymphoid areas of the spleen and kidney (Ellis 1981).

Serum antibodies, complement and CRP can significantly enhance phagocytosis and bacterial killing in both neutrophils and macrophages of fish. This suggests complement C3b and antibody Fc receptors are present on fish phagocytes (Kodama, et al. 1989; Ainsworth 1992; Kennedy-Stoskopf 1993). Receptors for complement have yet to be identified, but Fc receptors have been demonstrated in the nurse shark (Haynes, et al. 1988).

Nonspecific cytotoxic cells. Nonspecific cellular immunity in teleost fish is also mediated by a population of cells called non-specific cytotoxic cells (NCC). Elasmobranchs are capable of nonspecific cellular cytotoxic reactions, but cytotoxicity is mediated by macrophage-like cells instead of NCC (Haynes and McKinney 1991). The NCC of fish appear to be analogous to mammalian natural killer (NK) cells. Like NK cells, NCC are non-T cell, non-B cell, non-phagocytic lymphocytes that are nylon wool and plastic adherent/non-adherent, and irradiation resistant (Evans, et al. 1984; Tizard 1992). Morphologically, the NCC of fish are different from NK cells in that they are generally small, agranular lymphocytes, compared to NK cells which are large granular lymphocytes (Evans and Jaso 1992).

Mammalian cytotoxic T cells produce an antibody-dependent, cell-mediated cytotoxicity with an antibody determined specificity. Both NCC and NK cells differ from cytotoxic T cells in that they have the ability to spontaneously lyse tumor target cells, virus infected cells, and protozoan parasites without prior exposure or immunization. In addition, their lysis is not restricted by major histocompatibility complex antigens, they are able to lyse xenogeneic target cells, and there are no anamnestic responses (Evans and Cooper 1990). Similar to NK cells, NCC require cell-cell contact for target cell lysis, and they kill target cells by both apoptotic and necrotic mechanisms. However, it takes fewer NCC to kill an individual target cell, and it takes

NCC less time to kill, compared to NK cells . Unlike NK cells, NCC do not recycle. (Evans and Jaso 1992).

Fish NCC are found in the anterior kidney, spleen and peripheral blood. An antigen binding receptor has been identified and characterized on NCC using mAb. The receptor is a vimectin-like protein that recognizes a 40 kd protein determinant on the membranes of target cells. The receptor appears to be phylogenetically conserved because NK cells of rat and humans recognize the same 40 kd protein on target cells. Binding of the NCC receptor triggers events characteristic of signal transduction activity (Evans and Jaso 1992).

Inflammatory Responses

Inflammatory responses are dynamic nonspecific immune responses that occur at the sites of injuries caused by wounds or invasive organisms. In humans, the classic signs of inflammation include pain, swelling, redness and heat. The inflammatory response in fish is generally similar to that of mammals, although the response is less contained.

The general steps of inflammation include vasodilation and increased vascular permeability, leucocyte migration and removal of debris, and resolution and healing. Increased vasodilation and vascular permeability, resulting in swelling or edema, are the first step in the inflammatory responses of both fish and mammals (Suzuki and Iida 1992). This is followed by a biphasic leucocyte migration, with neutrophils arriving at the site of inflammation more quickly than macrophages. Chemical mediators control leucocyte migration, and complement factors, leucotriene B₄, and a lymphokine have been identified as chemotactic and chemokinetic factors for fish neutrophils (Suzuki and Iida 1992). Both neutrophils and macrophages act to remove debris by phagocytosis. The majority of phagocytosis occurs in the first 3-4 days of the inflammatory response. Leukocyte migration, infiltration and phagocytosis are followed by tissue repair (Suzuki and Iida 1992; Secombes 1996).

The acute inflammatory response of fish results in hemorrhagic liquefaction rather than contained suppuration (Ellis 1981). This response is more damaging to host tissues, and Ellis (1981) hypothesized fish may be able to respond in this way due to the greater regenerative

power of their tissues. For example, fish muscle tissue continues to divide through-out life, so damaged muscle cells are replaced by new cells rather than the scar tissue that forms in mammals.

If the inflammatory stimuli persists following the acute inflammatory response, a chronic inflammatory response may follow. Granuloma formation is a typical chronic inflammatory response in fish. Granulomas are organized collections of macrophages and fibrous tissue stroma. As the granuloma progresses, the macrophages aggregate and transform into epitheloid cells or multinucleate giant cells (MNGCs), and extensive melanization and fibrosis occurs. The granulomas may displace or isolate the surrounding host tissue resulting in tissue atrophy (Ellis 1981; Secombes 1996).

Hypersensitivity responses in fish is a controversial topic. Behavioral reactions comparable with type I hypersensitivity, or anaphylaxis, have been demonstrated in some teleost species but not others (Ellis 1981; Kennedy-Stoskopf 1993). Since fish do not have mast cells or IgE, and only very low detectable levels of histamine, the controversy is whether or not fish can truly experience anaphylaxis. Fish do have tissue eosinophilic granular cells which appear to be mast cell-like in ultra-structure and cytochemical studies (Ellis 1982; Kennedy-Stoskopf 1993). Type II (cytotoxic) hypersensitivity and type III (immune-complex) hypersensitivity have not been documented in fish, but are thought to occur because all of the necessary components are present (Ellis 1981; Kennedy-Stoskopf 1993). Type IV (delayed) hypersensitivity does occur in fish and is discussed further under cell-mediated immune responses.

Specific Immune Responses

Humoral Immune Responses

The specific humoral (antibody) response of fish is the best studied aspect of fish disease resistance. Although there are differences, the humoral immune response of fish shares some general features in form and function with that of mammals. The shared features include antibody effector mechanisms, basic immunoglobulin structure, and the cellular requirements of antibody induction (Kaattari and Piganelli 1996).

B lymphocytes. Antibodies are produced by the B lymphocytes. In mammals, lymphocytes are only called B lymphocytes, or B cells, if they synthesize and express membrane bound immunoglobulin (mlg) molecules on their cell surface. Upon stimulation, replicating B cells will either become memory cells or antibody secreting plasma cells. Plasma cells are termed "terminally differentiated" because they will not persist or self-renew. Terminal differentiation is accompanied by the loss of mlg as well as many other surface B-cell markers (Kincade and Gimble 1993). Fish lymphocytes do consist of two populations of cells, those with membrane bound immunoglobulin (mlg⁺) on their surface, and those without surface immunoglobulin (mlg⁻; Lobb and Clem 1982).

The B cells arise from the bursa of birds and the bone marrow of mammals. In fish, which lack bone marrow, the primary lymphoid organ is thought to be the anterior kidney. The lymphoid tissues of fish which contain the highest levels of mlg⁺ cells are the kidney, spleen and blood. The thymus of fish contains predominantly mlg⁻ cells, however very low levels of mlg⁺ B cells also occur (Kaattari 1992). The B cells found in the anterior kidney produce low affinity antibodies with a restricted repertoire of specificity, whereas the B cells found in the spleen and posterior kidney produce both high and low affinity antibodies with diverse specificities. It has been suggested that the restricted B cell population of the anterior kidney may be indicative of the anterior kidney's role as a primary lymphoid organ for the B cell, with more mature B cells residing in the secondary lymphoid organs (Kaattari and Irwin 1985; Kaattari 1992).

The B cells are activated by antigens, and antigens, based on their structure, are divided into two forms. These forms are T-independent (TI, polysaccharides) and T-dependent (TD, proteins). Most antigens, with a few exceptions, can be classed as one of these two forms (Kaattari and Piganelli 1996). The TI antigens are further classified into type I or type II. Most TI type I antigens are polyclonal B cell activators, which are directly mitogenic for B cells, regardless of antigen specificity, and they induce polyclonal antibody production (DeFranco 1993). Many of these compounds are components of bacterial cell walls, such as LPS. Like mammalian B cells, fish mlg⁺ cells have a mitogenic response to LPS (Sizemore, et al. 1984). Hapten-carrier experiments using LPS showed fish anti-hapten responses to TI antigens require

hapten-specific mlg^+ cells and accessory cells (monocytes). Anti-hapten responses to TD antigens required hapten specific mlg^+ cells, carrier-specific mlg^- cells, and monocytes (Miller, et al. 1985). The role of the mlg^- cells is discussed further below under cell-mediated immune responses. The role of the monocyte appears to be in providing a soluble factor with a function analogous to mammalian IL-1 (Vallejo, et al. 1992a).

Antibody responses. Upon immunization or first exposure to an antigen, fish produce specific antibodies with measurable affinities for the eliciting antigen. Depending on the route of antigen exposure, antibodies may appear in the serum, mucus and bile (Wilson and Warr 1992). The kinetics of the humoral response of bony fish can be broken down into three phases, a lag phase, exponential phase and decay phase. Circulating antibody levels reach their peak three to four weeks following the first exposure to the antigen. Upon a second exposure to an antigen, the lag phase is shorter, the response is accelerated, and higher antibody titers are produced (van Muiswinkel 1995). The anamnestic, or secondary response, of fish does differ from that of mammals. The differences are discussed below under memory responses.

Antibody effector mechanisms. The effector mechanisms of fish antibodies are the same as those of mammals. The effector mechanisms include neutralization, precipitation and agglutination, opsonization, and complement activation (Kaattari and Piganelli 1996). Neutralization occurs when specific antibodies block the function of an antigen by binding to receptors, active sites of enzymes, or toxigenic determinants. Precipitation and agglutination occur when antibodies cross-link antigenic molecules, molecules in solution result in precipitation and molecules attached to cell surfaces result in agglutination. Opsonization is when coating of bacteria, fungi or parasites with antibody leads to enhanced phagocytosis. Complement activation occurs via the classic pathway when antigen-antibody complexes result in activation of C1, the first component of the complement cascade (Sakai 1992; Tizard 1992; Kaattari and Piganelli 1996).

Immunoglobulin structure. Fish immunoglobulin is a high molecular weight molecule which is similar to mammalian immunoglobulin M (IgM). Although initially referred to as IgM-like,

the molecule is now generally referred to as IgM. Calling the molecule IgM is thought to be justified based on the size, structure, gene organization and physiochemical properties that the fish immunoglobulin molecule shares with mammalian IgM (Wilson and Warr 1992).

Fish immunoglobulin consists of equimolar amounts of larger (~72 KD) heavy chains (H) and smaller (~27 KD) light chains (L). The monomeric form of the molecule is H_2L_2 and contains two antigen binding sites. The IgM of elasmobranchs is a pentamer, like the IgM of mammals, and consists of 10 heavy and 10 light chains $(H_2L_2)_5$ and a total of 10 antigen binding sites. The IgM molecule of teleosts is a tetramer and consists of eight heavy and eight light chains $(H_2L_2)_4$ and a total of eight antigen binding sites (Wilson and Warr 1992; Kaattari and Piganelli 1996).

Antibody molecules are often described as having two different regions, an antigen-binding amino terminus (Fab) and a carboxy-terminal effector region (Fc). In the fish IgM monomer, the Fab portion of the molecule consists of the N-terminal portions of each of the H and L chains. Like mammalian IgM, fish IgM heavy and light chains consist of constant and variable regions. Fish IgM heavy chains have one variable region (VH) at the amino terminus followed by four constant regions (CH_1 , CH_2 , CH_3 and CH_4). Fish IgM light chains have one variable region (VL) at the amino terminus followed by one constant region (CL). The H_2L_2 monomer is held together by three disulfide bonds. The two H chains of the monomer associate so that the VC, CH_1 , CH_2 , CH_3 and CH_4 regions are aligned, with the chains held together by a disulfide bond between the CH_4 regions. Each of the two L chains in the monomer pair up with an H chain so that the VL and CL regions are aligned with the VC and CH_1 regions, and the chains are held together by a disulfide bond between the CL and CH_1 regions. The Fab portion of the molecule consists of the VL and CL regions of the L chains and the VH and CH_1 regions of the H chains. The Fc portion of the molecule consists of the CH_2 , CH_3 and CH_4 regions of the H chains (Wilson and Warr 1992; Kaattari and Piganelli 1996). The variable regions of the Fab portion of the molecule are what recognizes and binds to the antigen. Within each variable region there are three hyper-variable regions which are called complementarity determining regions (CDR1-3). The remaining four variable regions surrounding the CDRs are called framework regions (FR1-4) (Pilstrom and Bengten 1996).

The monomeric subunits of mammalian IgM are held together in their pentameric form by complete disulfide bond cross-linking. Complete cross-linking of the subunits is stringently required, and the molecules that are only partially cross-linked are targeted for digestion rather than secretion. In contrast, the disulfide cross-linking of the monomers of teleost IgM does not occur uniformly, and seems to be a means by which structural diversity is generated. Differential cross-linking results in varying combinations, depending on the species of fish, of tetramers, trimers, dimers and monomers that may be covalently or non-covalently associated (Kaattari and Piganelli 1996).

Isotypic diversity. Within a single species, immunoglobulins may be separable into classes, or isotypes, based on structural differences between the classes. Human immunoglobulin consists of nine different isotypes (IgM, IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgE and IgD). The differences in isotypes are due to differences in the constant regions of the H chains (Kaattari and Piganelli 1996). Teleosts appear to have limited isotypic diversity compared to that of mammals. Heavy chain isotypes have been deduced in some fish, but they are all IgM-like isotypes. In sharks and teleosts, the low molecular weight immunoglobulins are made up of the same monomers as the complete IgM molecule (Wilson and Warr 1992). An exception to this is seen in rays, which do appear to have a structurally distinct low molecular weight immunoglobulin (Pilstrom and Bengten 1996). Teleost light chains appear to consist of two isotypic variants similar to mammalian kappa and lambda chains (Wilson and Warr 1992).

The genes encoding immunoglobulins. The genes encoding H and L polypeptide chains of fish immunoglobulin are located at separate loci in the fish genome. Like mammalian immunoglobulin, the H chains of teleost and holostean fish are encoded by multiple genetic segments. Multiple variable (V) regions are located upstream of cluster of diversity (D) regions, followed by joining (J) regions. Further down at the most 3' end are the constant (C) regions (Pilstrom and Bengten 1996). The VH regions are encoded by V_H, D and J_H gene segments, and each of the C_μ (CH1 to CH4) regions are encoded by an exon. During B cell development, the V_H, D and J_H gene segments rearrange and fuse to form the VH region. In most vertebrates,

including teleost and holostean fishes, there are more copies of the diversity-generating V_H , D and J_H gene segments than there are of the CH region genes. This type of gene arrangement is referred to as a translocon arrangement (Warr 1995; Pilstrom and Bengten 1996). In contrast to the translocon arrangement, the heavy chain genes of elasmobranchs are organized into repeating clusters of one V_H , two D, one J_H and one $C\mu$ region (V_H , D_1 , D_2 , J_H , $C\mu$) which do not rearrange. This type of gene arrangement is referred to as a multicuster or multiple locus arrangement (Warr 1995; Pilstrom and Bengten 1996).

Unlike the heavy chain genes, the light chain genes of cartilaginous and bony fish are arranged in a similar manner to each other. The VL regions are encoded by V_L and J_L gene segments (the D region is absent), and the CL regions are encoded by a single exon. The V_L , J_L and CL regions are in a multicuster arrangement similar to that of the elasmobranch heavy chain genes. The light chain genes of cartilaginous and bony fish do differ in transcriptional orientations. In cartilaginous fish (Chondrichthyes), the V_L , J_L and CL regions are all transcribed in the same direction. In bony fish (Osteichthyes), the V_L region is transcribed in the opposite direction of the J_L and CL regions (Warr 1995; Pilstrom and Bengten 1996). It is not yet certain if fish possess light chain classes analogous to the kappa and lambda classes of mammals, but the fish light chain genes of individual species examined so far can be separated into distinct classes based on structural type (Warr 1995).

Immunoglobulin gene expression. In mammals, transcription of the immunoglobulin genes is regulated by nuclear factors. These nuclear factors are DNA binding proteins which recognize and bind to specific conserved nucleotide sequences, or motifs called promoters and enhancers. Immunoglobulin gene expression requires that the process of V(D)J rearrangement brings the B cell-specific promoter and enhancer elements in sufficiently close proximity to each other so transcription of a functional gene can be initiated. Immunoglobulin promoters are located upstream of each V region. Enhancers for Ig heavy chains are found in the D region and in the intron between the J_H and $C\mu$ regions, and 3' of the constant region genes (Warr 1995; Pilstrom and Bengten 1996).

Little is known about fish Ig transcriptional control compared to what is known in humans and mice. The minimal motif requirements for B cell promoter function are the TATA box and the octamer ATGCAAAT. These promoter motifs have been identified in the 5' flanking region of all fish genes examined so far, with the exception of elasmobranch IgH clusters, which instead have a decamer/nanomer motif like in the promoter region of mammalian T cell receptors. Inspection of sequences has identified enhancer motifs in many fish similar to those of mammals. Other promoters and enhancers of fish Ig genes have motifs similar to mammals, but their organization and arrangement differs. (Warr 1995; Pilstrom and Bengten 1996).

Secreted and membrane bound IgM. All immunoglobulins have the potential to exist as either the soluble secreted molecule (slg) found in blood, mucus and other body fluids, or the membrane-bound molecule (mlg) that acts as a receptor for antigen on the surface of B cells (Warr 1995). One of the distinguishing characteristics of B cell differentiation into antibody producing plasma cells is the loss of the mlg. While B cells express and make substantial quantities of both mlg and slg, upon stimulation and differentiation into a plasma cell, the cell stops expressing mlg and begins expressing slg almost exclusively. This switch is consistent with the function of the plasma cell, which is to generate a large pool of circulating antibodies (Wilson and Warr 1992; Max 1993).

In most vertebrates, the mlg is a slightly larger molecule than the slg due to an additional C-terminal hydrophobic segment in both H chains that anchors the protein in the membrane lipid. In the case of the μ chain, this transmembrane domain is encoded by two additional exons, TM1 and TM2. Alternate mRNA processing determines whether the membrane bound or secreted form of the IgM is expressed (Wilson and Warr 1992; Max 1993). In most vertebrates, the mRNA for the membrane form of the molecule is derived when the TM1 and TM2 exons are spliced into a cryptic donor splice site located at the 3' end of the CH4 exon. The TM exons encode 41 C-terminal residues, including 26 uncharged residues which are thought to span the membrane. The mRNA of the secreted form of the molecule does not include the TM1 and TM2 exons (Max 1993).

Fish also possess two additional transmembrane domain encoding exons. Chondrosteans use the same cryptic donor site as other vertebrates, but this site is lost in teleosts. As a result, the mRNA processing of teleosts differs from that of other vertebrates. In teleosts, the TM domains are spliced into a cryptic donor site located at the 3' boundary of the CH3 exon, resulting in elimination of the CH4 exon (Wilson and Warr 1992). The resulting truncated molecule is still able to function normally as an antigen receptor and mediate allelic exclusion (Miller, et al. 1994b).

Holosteans appear to be in a transition state between chondrosteans and teleosts. The two holostean species examined, bowfin (*Amia calva*) and longnose gar (*Lepisosteus osseus*), use both the CH3 and CH4 cryptic donor sites. In addition, the bowfin has a third cryptic donor site located in the middle of the CH3 exon which is used in the production of mIg encoding mRNA (Wilson, et al. 1995a; Wilson, et al. 1995b).

The B cell receptor (BCR). The B cell antigen receptor of mammals is composed of mIg and two accessory proteins. These accessory proteins, Ig- α (CD79a) and Ig- β (CD79b), form a covalently linked heterodimer complex that associates non-covalently with the mIg molecule. The Ig- α and Ig- β proteins are encoded by the B cell-specific genes *Mb-1* and *B29*, respectively. These accessory molecules are responsible for both surface expression of mIg and signal transduction of the B cell receptor (BCR; DeFranco 1993; Ryszczyn, et al. 1996).

Investigation of the fish BCR has only recently been done in channel catfish (Ryszczyn, et al. 1996). The mIg on the surface of catfish B cells was found to be non-covalently associated with accessory molecules. These molecules differed from those of mammals. Mammalian accessory molecules exist as one set of molecules in a Ig- α /Ig- β heterodimer, consisting of 34 and 40 KD polypeptides respectively in a 2:1 ratio with mIg (DeFranco 1993; Ryszczyn, et al. 1996). Instead of one set of accessory molecules, the catfish mIg appears to be associated with two sets of molecules of 64 and 70 KD that are distinct from each other. The 64 KD molecule appears to be a dimer of non-glycosylated 32 KD peptides, and the 70 KD molecule appears to be a heterodimer of a 45 KD glycosylated peptide and a non-glycosylated 25 KD peptide. Both

the 64 and 70 KD sets of accessory molecules are rapidly phosphorylated on tyrosine residues following mlg cross-linking, indicating fish B cells use a signal transduction pathway similar to mammals (Ryszczyn, et al. 1996).

The generation of antibody diversity. The antibodies of fish and other endothermic vertebrates are of much lower affinity and diversity than those of higher vertebrates (Warr 1995). Although information on the generation of antibody diversity in fish is limited due to the fact that few species have been investigated, the mechanisms responsible for generating antibody diversity in mammals are also thought to be present in fish. Six mechanisms responsible for generating immunoglobulin diversity in mammals have been identified, they include combinatorial diversity, junctional imprecision, junctional diversity, gene conversion, heavy and light chain pairing, and somatic mutation (Tizard 1992; Warr 1995).

Combinatorial diversity, the first mechanism for generating antibody diversity, is the random selection of the individual V, D and J gene segments that will rearrange and fuse to form the V region (Tizard 1992; Warr 1995). Combinatorial diversity occurs with the IgH locus of teleost fish, which have a translocon gene arrangement, but it is generally thought that the multicenter arrangement of the elasmobranch IgH loci and the IgL loci of all fish will restrict diversity generated by combinatorial means (Warr 1995). The multicenters are closely linked however, and since the V_L segments are transcribed in the opposite direction of the J_L and C_L segments, it has been suggested that rearrangements between clusters could occur by inversion, as seen in the murine κ -locus (Pilstrom and Bengten 1996).

The number of different V, D and J gene segments a species has in its germline determines how many different V, D, J joining combinations are possible. In humans and mice there are over 100 germline V_H genes. These genes are grouped into seven families in humans and 14 families in mice (Warr 1995). In comparison, channel catfish have over 100 V_H genes grouped into six families, rainbow trout have 9 V_H families, Atlantic cod (*Gadus morhua*) and goldfish have at least three V_H families (Warr 1995). Less information is available for the J and D regions of fish. The only fish J_H regions that have been cloned and mapped from genomic DNA are those of channel catfish, which have been shown to have nine J_H regions compared to

humans and mice which have four and six J_H regions respectively. Provisional estimates of D_H regions and J_H regions of fish species have been made using cDNA analysis, which shows rainbow trout have at least 6 J_H regions and ten D_H regions, and Atlantic cod (*Gadus morhua*) have at least two J_H regions (Warr 1995; Pilstrom and Bengten 1996).

Less is known about fish light chain regions, but Southern blot analyses have shown that the three teleost fish studied (channel catfish, rainbow trout and Atlantic cod) all appear to have about 20 V_L regions. Since the V_L and J_L regions of fish are in multicusters, there must also be about 20 corresponding J_L regions (Pilstrom and Bengten 1996). Based on these estimates, it does not appear that teleosts are lacking in V, J and D region diversity.

Junctional imprecision and junctional diversity are the second and third mechanisms responsible for generating antibody diversity. Junctional imprecision is the imprecise joining of the V, D and J gene segments. Junctional diversity is a result of enzymatic addition and removal of bases at the junctions during joining of the V, D and J gene segments (Tizard 1992; Warr 1995). These mechanisms of diversity have not been well studied in fish, but they have been observed to operate in the heavy chains of rainbow trout and Atlantic salmon (Pilstrom and Bengten 1996). Gene conversion, the fourth mechanism for diversity, is the non-reciprocal exchange of sequences from non-functional V pseudogenes to functional V regions. This mechanism is a major means of generating antibody diversity in chickens and rabbits. It may also be important in other mammalian species, and it is possible it occurs in fish (Pilstrom and Bengten 1996). The fifth mechanism for diversity, heavy and light chain pairing, is the random selection of expressed heavy and light chain pairs in a B cell. There is no reason to think random selection of expressed chains does not occur in fish.

The final mechanism for generating antibody diversity, somatic mutation, is the introduction of point mutations in the joined V, D, J regions which occur after antigen stimulation of the B cell (Tizard 1992; Warr 1995). In mammals, somatic hypermutations occur in the B cell blasts before they differentiate into antibody producing plasma cells. The hypermutations occur most commonly in the gene regions that code for the CDRs of the molecule. The result of these hypermutations in the CDRs is an increase in the affinity of the antibodies for a particular antigen

as the immune response progresses (Pilstrom and Bengten 1996). This increased antibody affinity, termed affinity maturation, is what is responsible for the characteristic strong antibody responses in mammals (Warr 1995). An affinity maturation like that of mammals does not occur in fish and other poikilotherm vertebrates, and this initially led researchers to believe it was because somatic mutation did not occur. Recent studies have shown, however, that somatic mutations do contribute to antibody diversity in amphibians and sharks (Wilson, et al. 1992; Hinds-Frey, et al. 1993). Somatic mutation in teleosts has not yet been investigated, but will likely show similar results (Pilstrom and Bengten 1996).

Secondary immune responses. The primary immune response is one that occurs following first exposure to an antigen. The secondary immune response occurs following a second or subsequent exposure to the same antigen and is specifically altered from the primary response (Tizard 1992). The secondary response is also called the anamnestic or memory response. The hallmarks of the mammalian memory response to protein antigens include enhanced production of antibody, isotype switching, and affinity maturation. The enhanced antibody production is seen as a faster and higher rise in antibody titer than in the primary response. Isotype switching is a switch from IgM to lower molecular weight Ig. Affinity maturation is a shift to higher affinity antibodies by somatic mutation (Wilson and Warr 1992). Each of these mammalian elements are discussed in regard to fish below.

The first element of the mammalian memory response is a faster and higher rise in antibody titer than the primary immune response. Secondary antigen exposure in fish also results in an increase in antibody titer. The magnitude of the increase in titer is lower in fish than in mammals. In mammals the secondary antibody response is up to 100 times higher than the primary response, while in rainbow trout and carp the secondary response is 10-20 higher than the primary response (Arkoosh and Kaattari 1991; van Muiswinkel 1995). In mammals, increased antibody titer of the secondary response is due to a logarithmic increase in the concentration of monomeric immunoglobulin (IgG). In fish, which only have multimeric immunoglobulin, the increase in titer of the secondary antibody response is due to an increase in the concentration of IgM. Because of the higher magnitude of the response, most researchers

view the mammalian immune response as superior to that of fish. In contrast, Kaattari (1994) suggests the mammalian response is higher not because it is a superior response, but simply because it takes more monomeric antibody molecules to have the same effectiveness as multimeric antibodies.

In both mammals and fish, the increase in antibody titers has been shown to be due to an increase in the number of specific antibody producing B cells rather than simply an increase in antibody secretion by existing B cells. In rats, the enhanced secondary response is due to both an increase in the number of responsive B cell precursors and clonal proliferation of these B cell precursors. Limiting dilution analysis has shown memory development in rainbow trout is due exclusively to the generation of an enlarged pool of B cell precursors (Arkoosh and Kaattari 1991). There is no difference in the clonal proliferation of the primary and secondary responses to antigen, suggesting the memory cell precursors are not physiologically different from the primary population with respect to proliferation potential. This means that although a distinctive memory response can be detected in fish, a distinctive memory cell cannot be distinguished (Arkoosh and Kaattari 1991; Kaattari 1994; Kaattari and Piganelli 1996).

Another difference between the primary and secondary response of mammals is an increased sensitivity to antigen in primed animals. The secondary immune response requires exposure to a much lower dose of antigen compared to the primary immune response. This has also been observed in rainbow trout (Arkoosh and Kaattari 1991; Kaattari 1994). Trout which were primed with an immunogenic dosage (100 µg) of the T-dependent antigen trinitrophenylated keyhole limpet hemocyanin (TNP-KLH) had an enhanced secondary antibody response to a much lower dosage (20 µg) of TNP-KLH. This low dosage of TNP-KLH was shown to be non-immunogenic in unprimed fish (Arkoosh and Kaattari 1991). The increased sensitivity to antigen could be due to a physiological difference between the memory B cell and the naive B cell, though it is more likely due to an expansion of high affinity B cell clones over low affinity clones (Kaattari 1994).

The second element of the mammalian memory response is an isotype switch from IgM to IgG. Heavy chain isotypes have been deduced in some fish, but they are all IgM-like isotypes

(Wilson and Warr 1992). Relative shifts in isotype concentrations have been observed in fish, but there is no evidence isotype switching occurs (Kaattari and Piganelli 1996). Since fish Ig isotypes are all multimeric, an isotype switch may not be necessary. Furthermore, isotype switching of mammalian B cells to full time IgG production may just be an economic way to produce the high amounts of IgG, rather than a universal requirement for the generation of memory in all animals (Kaattari 1994).

The third element of the mammalian memory response is affinity maturation, which is a shift to high affinity antibodies due to somatic mutations. There is evidence that somatic mutation occurs in fish, at least in elasmobranchs (Hinds-Frey, et al. 1993). Since somatic mutation occurs in fish, they should have the capacity to generate a variety of binding sites that could lead to affinity maturation, but they do not appear to do so (Kaattari and Piganelli 1996). One possible explanation is affinity maturation in fish is being inappropriately assessed because the methods being used are suitable for monomeric Ig but not multimeric Ig (Kaattari 1994). Measurements of affinity maturation are usually done using equilibrium dialysis, which measures the intrinsic affinity of the individual binding site. Functional affinity is a measure of the affinity of the entire antibody molecule, as opposed to the intrinsic affinity of a single binding site. Mammalian IgG and IgM antibodies to the same antigen have been shown to have comparable intrinsic affinities, but dramatically different functional affinities (Kaattari 1992). Barely detectable shifts in intrinsic affinity can result in a 100-fold increase in the functional affinity (avidity) of a multimeric molecule. Kaattari (1994) suggests that the intrinsic affinity shifts, or the somatic mutations which cause them, may not be a requirement for the memory response, but rather a requirement for an effective monomeric antibody response specific for mammals. In order to compete for circulating antigen, monomeric antibodies must have intrinsic affinities that are high enough to compete with the functional affinities of multimeric antibodies.

It seems clear that the question of whether or not affinity maturation occurs in fish cannot be accurately answered until a more appropriate means of assessing fish multimeric antibody affinity is employed (Kaattari 1992). Kaattari has recently reported on an ELISA technique that estimated the relative affinities of antibody populations in the serum of rainbow trout. The results

showed a greater than 10-fold increase in the average antibody affinities during an immune response to TNP-KLH, which appeared to be due in part to the emergence of new high affinity antibodies (Kaattari 1994).

Many researchers have concluded that a memory response does not occur in species that do not appear to have the mammalian hallmarks of memory (Wilson and Warr 1992). Fish, which lack isotype switching and for whom affinity maturation has not yet been adequately demonstrated, are one of the groups not fitting the mammalian paradigm. Kaattari (1994) warns that using mammalian-based definitions and methods of assessment could potentially lead to errors in the interpretation of immunological phenomenon in phylogenetically distinct species. Instead, he suggests that the memory response should be defined by identifying the most essential elements, keeping in perspective the species-specific nuances. A simplified definition of immunological memory, which is phylogenetically unbiased and may be more appropriate, is that a memory response is one that is distinctive in form and function from a primary response and has enhanced immunoreactivity (Kaattari 1994).

Cell-Mediated Immune Responses

Traditionally, specific immune responses that are not due to antibodies are thought of as being cell mediated immune responses. Cell mediated immunity is immunity that is mediated by T-lymphocytes and macrophages, and can be conferred on an animal by adoptive transfer of live cells (Tizard 1992). In actuality, most specific immune responses require T lymphocytes. Only antibody responses to T-independent antigens are due to direct stimulation of the B cells, and these T-independent antigens do not generate immunological memory (Arkoosh and Kaattari 1991). Antibody responses to T-dependent antigens, which do generate a memory response, require stimulation of the B cells by T-helper cells (Arkoosh and Kaattari 1991; Tizard 1992).

T lymphocytes. The T lymphocytes, or T cells, originate in the thymus and are the mediators of cell-mediated immune responses. In mammals, different functions are regulated by different subsets of T cells. The helper T cells (T_H cells) enhance immune responses. The cytotoxic/suppressor T cells (T_c or T_s cells) destroy foreign and abnormal cells, and down

regulate the responses of other B and T cells (Kennedy-Stoskopf 1993). Mammalian T cells subsets can be differentiated from each other by the presence of the different antigens on their surface. The T_h cells have the CD4 surface antigen and the T_{cyt} cells have the CD8 surface antigen. Consequently, the $CD4^+CD8^-$ T_h cells are sometimes referred to as CD4 cells, and the $CD4^-CD8^+$ T_c cells are referred to as CD8 cells.

In mice, and presumably other mammals, the T_h cells can be divided into three subgroups, designated T_{h1} , T_{h2} , and T_{h0} , based on the cytokines they secrete in response to antigens. The T_{h1} cells secrete interleukin-2 (IL-2), IL-3, INF- γ , tumor necrosis factor β (TNF- β) and granulocyte-macrophage colony-stimulating factor (GM-CSF), and they respond optimally to antigen presented by B cells. The T_{h1} cells secrete their cytokines soon after antigen stimulation. These cytokines activate macrophages, promote B cell proliferation and polyclonal IgG secretion, but they do not stimulate specific antibody formation. In some circumstances, T_{h1} cells may be cytotoxic and suppress antibody secretion. The T_{h1} cells also mediate cell-mediated (type IV) hypersensitivity (Tizard 1992). The T_{h2} cells secrete IL-3, IL-4, IL-5, IL-6 and GM-CSF, and they respond optimally to antigen presented by macrophages. The T_{h2} cells do not secrete their cytokines until several days after antigen exposure. These cytokines stimulate B cell proliferation and polyclonal IgM, IgA and IgE secretion, but T_{h2} cells are not cytotoxic and they do not mediate type IV hypersensitivity reactions (Tizard 1992). The T_{h0} cells in mice are a population of T cells that secrete cytokines representative of both T_{h1} and T_{h2} cells. The T_{h0} cells may be precursors of T_{h1} and T_{h2} cells, or they may be cells in transition between the two populations. There is some evidence that T cells can switch phenotype from T_{h1} to the T_{h2} . Although two distinct T_h populations can be phenotypically identified in mice, this is not the case with other domestic mammals, in which the $CD4^+$ cells produce multiple cytokines, including IL-2, IL-4 and INF- γ (Tizard 1992).

Fish are also presumed to have T lymphocytes. The T cells of fish are IgM $^+$ lymphocytes which are found predominately in the thymus. Migration of thymocytes to peripheral lymphoid organs has been demonstrated in rainbow trout using radiolabeling experiments, which showed that twice as many thymocytes migrated to the spleen as to the kidney (Tatner 1985). The study

of fish T cells has been hindered by the lack of a T cell marker, but T cell functions like those of mammalian T_h and T_c cells have been demonstrated in fish using *in vivo* and *in vitro* measures of histocompatibility and cell-mediated immunity (Chilmonczyk 1992).

T cell markers. Monoclonal antibodies (mAb) against mouse and human B and T cell surface antigens are not cross-reactive with fish lymphocytes (Miller, et al. 1987). Monoclonal antibodies have been made which recognize fish IgM and these have been used to identify and separate the mlg^+ B cells from the mlg^- lymphocytes. Enrichment for B cells from peripheral blood using mAb and panning techniques have resulted in separations of mlg^+ fractions which contained 90% putative B cell lymphocytes, with the rest being macrophages and neutrophils. The mlg^- fractions contained 30% putative T cell lymphocytes, with the rest being predominately thrombocytes (Manning and Nakanishi 1996).

Monoclonal antibodies have also been generated against fish T cells. A mAb, designated 13C10, has been generated against catfish peripheral blood leucocytes (PBL). The 13C10 reacts with mlg^- , but not mlg^+ lymphocytes. However, the mAb is not specific for T cells and is also reactive with thymocytes, neutrophils, thrombocytes, hepatocytes and brain cells. The antibody was shown to react with three proteins of 155, 110 and 70 KD on thymocytes and PBL (Miller, et al. 1987). Two other mAbs, designated C2-3a and C2-4a, were generated against PBL and shown to be reactive with PBL Igm^- lymphocytes (Ainsworth, et al. 1990). It was not determined if these mAbs were reactive to cell types other than those in PBL, and the antigens that the mAbs were reacting with were not characterized. A mAb called WCL9 was produced against thymocyte membrane molecules from carp (Rombout, et al. 1997). This mAb appears to recognize early thymocytes. The WCL9 reacts with two surface proteins of 155 and 200 KD (Rombout, et al. 1997). Another mAb, designated CfT1, has been recently generated against catfish thymocytes and Igm^- lymphocytes (Passer, et al. 1996). This mAb is reactive with thymocytes, a sub-population of lymphoid cells in blood and lympho-hemopoietic tissues (putative T cells), and a T cell line. The CfT1 is not reactive with erythrocytes, thrombocytes, myeloid cells, B cells or macrophage cell lines. The antibody was shown to be recognizing a non-glycosylated 35 KD cell surface protein. To date, CfT1 appears to show the most promise as a specific T cell

marker; however, it is not known if this mAb is recognizing all T cells, or a sub-population of T cells.

***In vivo* measurements of cell-mediated immunity.** The *in vivo* measurements of cell-mediated immune responses in fish include measurements of histocompatibility responses and hypersensitivity responses. Histocompatibility is determined by assessing the immune response to allografts (Manning and Nakanishi 1996). An allograft is a tissue transplant from one member of a species to another genetically distinct member of the same species (Hansen, et al. 1993).

Allografts in fish, usually skin or scales, are rejected in a manner similar to that of mammals. There is no evidence of antibody involvement in the graft rejection process (Manning and Nakanishi 1996). Lymphocytes and macrophages are the host cells that invade the graft. Teleost fish have an average acute rejection time of 14 days. Agnatha and elasmobranch fish have a chronic rejection response, taking an average of 30 days. Second-set grafts are rejected more rapidly than first-set grafts, indicating a memory response (van Muiswinkel 1995).

In addition to the host versus graft response described above, graft versus host reactions have also been described in fish. In this reaction, the grafted cells react against the host. The graft versus host reaction was seen when donor cells from triploid carp were injected into tetraploid host fish, obtained by crossing the carp with gold fish. The reaction ensued two weeks following the injection and involved damage to skin, liver and lymphoid organs, eventually resulting in death (Manning and Nakanishi 1996).

Delayed (type IV) hypersensitivity reactions (DTH) occur when certain antigens are injected into the skin of sensitized animals. The inflammatory skin reaction, called DTH because it takes 24 to 48 hours to reach full intensity, is due to the interaction between the antigen and sensitized T cells (Tizard 1992). Fish exhibit typical DTH reactions against *Mycobacterium* and parasitic antigens (Manning and Nakanishi 1996).

***In vitro* measurements of cell-mediated immunity.** In mammals, T_h cells function in the antigen stimulation of B cells to produce antibody to TD antigens. This can be measured *in vivo* by looking at hapten-carrier effects on the production of antibodies against TD antigens (Ellis

1982). Hapten-carrier effects have also been demonstrated in fish. Like mammals, the response requires carrier-specific cooperation by mlg^- lymphocytes identified as putative T cells (Miller, et al. 1985).

Proliferative responses to B and T cell mitogens can also be measured *in vivo*. In mammals, the plant lectins phytohemagglutinin (PHA) and concanavalin A (Con A) are specific for carbohydrate moieties on T cells. Appropriate doses of these T cell mitogens induces the proliferation of T cells but not B cells. Mitogens for B cells, such as LPS, result in the proliferation of B cells but not T cells (Manning and Nakanishi 1996). Fish also have the ability to respond to T and B cell mitogens. Fish thymocytes and mlg^- lymphocytes respond to Con A and PHA, but only in the presence of monocyte accessory cells (Miller, et al. 1985; Manning and Nakanishi 1996). Fish mlg^+ lymphocytes respond to LPS in the presence of monocyte accessory cells (Miller, et al. 1985). However, fish mlg^- cells also respond to LPS though the response is much lower. The apparent response of mlg^- cells to LPS may be due to contaminating mlg^+ positive cells that were not removed from PBL by panning, or to a small population of B cells residing in the thymus (Manning and Nakanishi 1996).

An *in vitro* test for allogeneic differences is the mixed leucocyte reaction (MLR). In mammals, an MLR occurs when CD4^+ T cells respond to foreign (non-self) MHC class II molecules on the surface of the stimulating cell. In most cases, the stimulating cell is a monocyte, macrophage or B cell. Interaction of the T cell with the stimulator cell causes stimulation of the T cell resulting in clonal proliferation and secretion of IL-2. If cells from two histoincompatible animals are mixed, the T cells of each animal will respond to the stimulator cells of the other in a reaction known as a two-way MLR. An MLR can be made one-way by preventing DNA synthesis in one set of cells using, for example, X-irradiation treatment. (Manning and Nakanishi 1996). An MLR response has been observed in agnathan, holostean and teleost fish (Stet and Egberts 1991). The cellular requirements of a one-way MLR were determined for catfish (Miller, et al. 1986). The results showed only mlg^- T cells were able to respond in the MLR, and monocytes were required as accessory cells. Monocytes, mlg^+ B cells and T cells could all function as stimulator cells.

Effects of thymectomy. In mammals, removal of the thymus from neonatal animals has a great effect. The effects of thymectomy in adults are initially not as great due to the presence of peripheral T cells; however, immunodeficiencies begin to occur as peripheral T cells begin to disappear (Manning and Nakanishi 1996). Complete removal of the thymus of fish is very difficult due to its contact with the pharyngeal epithelium. Because of this, thymectomy has only been accomplished in juvenile and adult fish. Removal of the thymus from young rainbow trout causes delays in allograft rejection. Thymectomy has no effect on allograft rejection when performed on adult fish (Chilmonczyk 1992).

Cytokines. Cell-mediated immune responses are mediated not only through direct cell-to-cell contact, but also through the release of soluble factors by cells (Manning and Nakanishi 1996). The regulatory proteins and glycoproteins released by cells are referred to as cytokines (Tizard 1992). Cytokines are simple proteins less than 30 KD that act as signaling molecules within the immune system (Secombes, et al. 1996). Fish produce a number of cytokine-like proteins. Most of these proteins have been identified using assays for biological activity, biological cross-reactivity, and antigenic cross-reactivity (Secombes, et al. 1996). Using these assays, soluble factors akin to mammalian IL-1, IL-2, IL-3, IL-4, IL-6, IFN- γ , tumor necrosis factor α (TNF- α) and transforming growth factor β , (TGF β ,) have been describe in fish (Manning and Nakanishi 1996; Secombes, et al. 1996).

Interleukin 1 is produced mainly by activated macrophages, but also a number of other cell types. It is an essential initiating factor for immune and inflammatory responses and its many functions include the activation of T cells and, as a cofactor with IL-2, the activation of B cells (Tizard 1992). Fish cells can produce an IL-1-like substance which can substitute for the requirement of monocyte accessory cells in *in vitro* immune responses, and catfish PBL can recognize and respond to human IL-1, suggesting a conserved IL-1 receptor (Manning and Nakanishi 1996; Secombes, et al. 1996).

Interleukin 2 is produced by CD4+ T cells in response to appropriately presented antigen or to some mitogens. It is an essential component of the immune response and its many

functions include inducing proliferation of activated T and B cells, inducing B cell Ig synthesis, and inducing proliferation and cytotoxicity of T cells and NK cells (Tizard 1992). In fish, a soluble factor with IL-2-like activity has been detected following T cell activation *in vitro* (Caspi and Avtalion 1984), and is expressed constitutively in an immortal catfish T cell line (Miller, et al. 1994a). A putative IL-2 gene has also been identified and cloned from flatfish (Tamai, et al. 1994).

Interleukin 3, IL-4 and IL-6 are produced by activated CD4⁺ cells. Interleukin 3 induces Ig secretion by B cells, IL-4 stimulates growth and differentiation of B cells, and IL-6 promotes the final maturation of B cells and Ig synthesis (Tizard 1992). In fish, a factor with IL-4-like activity is produced by an immortal catfish T cell line (Miller, et al. 1994a), and IL-3 and IL-6 have been detected in virus infected carp and rainbow trout using cross-reactive antibodies to mammalian cytokines (Ahne 1994). The biological activity of these three cytokines has not yet been investigated in fish (Manning and Nakanishi 1996).

Interferon γ is produced by T cells and NK cells after exposure to IL-2. It is a macrophage activating factor (MAF), and a macrophage migration inhibition factor (MIF). In fish, soluble factors with INF- γ -like MAF activity have been detected in supernatants of mitogen stimulated rainbow trout leucocytes (Graham and Secombes 1988; Graham and Secombes 1990), and an MIF can be demonstrated in elasmobranchs, holosteans and teleosts (Manning, et al. 1982; Manning and Nakanishi 1996; Secombes, et al. 1996).

Tumor necrosis factor α (TNF- α) is produced by macrophages and is involved in cell-mediated cytotoxic reactions. In fish, TNF- α has been reported in rainbow trout macrophages, it has been detected in virus infected carp and rainbow trout using cross-reactive antibodies to mammalian cytokines (Ahne 1994), and it has been shown that rainbow trout leucocytes and macrophages can respond to human recombinant TNF- α , suggesting a conserved TNF- α receptor (Manning and Nakanishi 1996; Secombes, et al. 1996).

Transforming growth factor β_1 (TGF β_1) is produced by platelets, activated macrophages, T cells and B cells (Tizard 1992). It is a potent deactivator of mammalian macrophages, and down regulates reactions initiated by MAF. Following activation with MAF, rainbow trout

macrophages can be down-regulated with bovine TGF β_1 , suggesting a conserved TGF β_1 receptor. In addition, a cDNA sequence for TGF with a 68% predicted amino acid homology to mammalian TGF β_1 has been obtained from rainbow trout (Manning and Nakanishi 1996; Secombes, et al. 1996).

Major histocompatibility complex (MHC). An allograft results in an immune response by the recipient which may lead to the rejection and destruction of the graft. The intensity of the immune response depends on how genetically related the donor and recipient are to each other. This immune response is initiated when the host T cells recognize foreign antigens on the surface of the transplanted cells. These antigens are encoded by genes which are part of a cluster of closely linked loci in the genome. Because of its central role in tissue compatibility, this set of loci is called the major histocompatibility complex (MHC). The MHC is responsible for controlling the cellular interactions that lead to an immune response (Tizard 1992; Hansen, et al. 1993).

All mammals have an MHC in their genome. The MHC is divided into three classes of genes. The class I genes encode glycoproteins expressed on the surface of all nucleated cells. The class II genes encode glycoproteins found only on the surface of lymphocytes and bone marrow derived-phagocytic cells. The class III genes encode a variety of proteins involved in the immune response including complement factors, TNF- α , and the 70 KD heat shock protein (Tizard 1992; Hansen, et al. 1993).

The mammalian MHC class I molecules consist of a 45 KD glycopeptide chain, called α , that is non-covalently linked to a 12 KD protein called β 2-microglobulin (β 2-m). The β 2-m is encoded by a gene outside of the MHC. The MHC class I antigens act as receptors for peptides derived from endogenously synthesized proteins. Endogenously synthesized foreign proteins occur, for example, in virus infected cells and tumor cells. The MHC class I antigens present these endogenous peptides to CD8 $^+$ T cells. The result is destruction of the infected cell by T cell-mediated cytotoxicity (Tizard 1992; Manning and Nakanishi 1996).

The mammalian MHC class II molecules are heterodimers consisting of two non-covalently linked glycoprotein chains called α and β . The α -chains have a MW of 30-34 KD and

the β -chains are about 26-29 KD depending on the locus involved. The MHC class II antigens act as peptide receptors on the surface of antigen presenting cells (APC). The MHC class II antigens present peptides derived from exogenously acquired proteins to CD4+ cells, which results in T cell-mediated help. A T_h cell cannot recognize a foreign antigen unless it is presented by an MHC class II antigen (Tizard 1992; Manning and Nakanishi 1996).

The initial identification of an MHC in mammals was done using allograft rejection reactions and MLRs (Stet and Egberts 1991). These two tests have also been used to support the existence of an MHC in teleosts. The acute allograft rejection of teleosts implies the presence of MHC class I antigens and the strong MLR implies MHC class II antigens (Kennedy-Stoskopf 1993). Attempts to confirm that fish have a MHC using RNA and DNA probes designed from known MHC nucleotide sequences of higher vertebrates, and using antibodies against mammalian and avian MHC molecules have been unsuccessful (Manning and Nakanishi 1996).

The proof came when Hashimoto *et al.* (1990) succeeded in isolating MHC genes from carp using the polymerase chain reaction (PCR). Since then, PCR has been used to isolate and sequence a number of genes representing all classes of MHC in both teleosts and elasmobranchs, but not in agnathans (Manning and Nakanishi 1996).

Because elasmobranchs, chondrosteans and agnathans exhibit chronic or subacute allograft rejection and all but agnathans exhibit weak MLRs, it has been suggested that these groups of fish lack an MHC and reject foreign antigen non-specifically (Kennedy-Stoskopf 1993). However, MHC genes have been identified in sharks, which suggests the lack of acute graft rejection in elasmobranchs is not due to the lack of MHC, and the presence of MHC genes is not always correlated with acute graft rejection (Manning and Nakanishi 1996).

Structurally, fish MHC genes are characterized by short introns, and their amino acid sequence homology with mammalian MHC molecules is only 40%. However, the overall organization of fish MHC genes is very similar to mammals. It has not yet been determined what chromosome the fish MHC genes are on, or if the loci are clustered in one complex. The MHC genes identified in fish have been cloned from cDNA libraries, indicating the MHC genes are being transcribed into mRNA, but the molecules encoded by the fish MHC genes have not been

identified, and their function in antigen presentation has not been determined (Dixon, et al. 1995; Manning and Nakanishi 1996). Expression of mRNA encoding a putative MHC class Ia molecule in rainbow trout was detected in the spleen, thymus, kidney, heart, intestine, brain and liver. The ubiquitous expression pattern of the gene was consistent with that of the MHC class Ia gene of mammals (Hansen, et al. 1996).

The T cell receptor (TCR). The T cell antigen receptor of mammals includes a disulfide-bonded heterodimer of either an α and β chain (α/β), or a γ and δ chain (γ/δ ; Hedrick and Eidelman 1993). These are encoded by genes specifically expressed and rearranged in the T cells in a manner similar to the Ig molecules of B cells. The heterodimer is part of a receptor complex that also includes a set of invariant glycoproteins collectively called CD3, and either the single-chain CD4 glycoprotein or the disulfide-bonded CD8 heterodimer (Tizard 1992). The T cells expressing the TCR α/β complex recognize specific antigen that is bound to, and presented by MHC class I or class II molecules on the surface of APC cells (Hedrick and Eidelman 1993). The process of antigen recognition by T cells expressing the TCR γ/δ complex is not well understood, but it is probably not MHC restricted. In mice and humans, the γ/δ TCR receptor is found on immature T cells, which make up about 1 to 3% of the PBL (Tizard 1992).

Like the MHC of fish, a molecule representing a fish TCR has not yet been identified, but the use of PCR strategies has led to the successful cloning of cDNA segments encoding putative TCR α and β -chains in rainbow trout, Atlantic salmon and horned shark (*Heterodontus francisci*). Analysis of the cDNA clones shows extensive sequence diversity in the variable regions of the molecules, which is due to both rearrangement and somatic diversification mechanisms (Manning and Nakanishi 1996). Specific expression of TCR β -chain mRNA was detected in rainbow trout thymocytes and splenocytes (Partula, et al. 1995).

Antigen processing and presentation. In mammals, T cells are able to recognize "non-self" antigens through a process that metabolically modifies these antigens to molecular forms expressed on the surface of accessory APC, such as macrophages. Antigen processing

is the cellular and biochemical processes that occur in the APC and lead to the exposure of epitopes. Antigen presentation is the subsequent interaction of the processed antigen with MHC molecules and the expression and presentation of the antigen in the context of MHC on the surface of the APC (Vallejo, et al. 1992a).

The proper processing of antigen requires that it be presented in an appropriate form to antigen sensitive T cells. Exogenous antigen is phagocytized and broken into short peptides of 12-24 amino acids by acidic proteases in the macrophage lysosome. These fragments bind to specific MHC class II antigens and are carried to the cell surface and presented by the MHC II antigens so they can be recognized by the TCR of CD4⁺ helper T cells. Endogenous antigens are not phagocytized by cells, but are "non-self" antigens originating in the cell itself. An example of endogenous antigens are proteins made by virus infected cells. These antigens are degraded by non-lysosomal proteases into short peptides of 8-9 amino acids and bound to transport proteins and carried across the endoplasmic reticulum where they are linked to a specific MHC class I antigens. The processed antigens are transported to the cell surface and presented by the MHC class I antigens so they can be recognized by the TCR of CD8⁺ cytotoxic T cells (Tizard 1992; Germain 1993).

The importance of antigen processing and presentation in the generation of immune responses to TD antigens has been clearly demonstrated in fish using *in vitro* studies. As in mammals, antigens are processed and presented by APC, such as macrophages and monocytes, to specific lymphocytes in an apparent MHC restricted manner. The antigen processing has been shown to involve proteolysis, which is presumed to occur in APC lysosomes. Studies of antigen processing in catfish reveal hallmarks of the exogenous pathway of antigen presentation (Vallejo, et al. 1992a).

Specific cytotoxic T cells. Cytotoxic cells with activities similar to mammalian cytotoxic T cells have been described in fish, but they have not been formally identified. These cells react against modified self cells or against allogeneic cells. The response against modified cells suggested MHC restriction was involved, similar to the MHC class I restriction of mammals.

The assays used in higher vertebrates to generate CD8+ cytotoxic T cells *in vitro* have apparently not yet been performed in fish (Manning and Nakanishi 1996).

Immunomodulators of Fish

Temperature

Ambient temperature influences the kinetics of metabolic processes in all poikilothermic vertebrates, including fish. As a result, environmental temperature has a tremendous influence on the immune responses of fish (Rijkers 1982). Generally, the optimal immune response occurs at temperatures corresponding to the normal summer temperatures of the species being considered. The optimal temperature will vary considerably depending on if the fish is a cold-water, temperate or warm water species (Rijkers 1982). Immunologically permissive temperatures are those at which optimal immune responses occur. Immunologically non-permissive temperatures are temperatures below the permissive range, but still within the physiological range of the species. Non-permissive temperatures tend to be immunosuppressive (Manning and Nakanishi 1996).

The temperature dependence of the fish immune response is important because it has been shown that seasonal variations in the incidence of infectious diseases are correlated to seasonal changes in water temperatures. This is seen most clearly in the spring and fall when rising or falling water temperatures result in more favorable temperatures for the growth and replication of some infectious agents (Rijkers 1982). Fish are most vulnerable at these times because their immune system has either not yet been built up, or is beginning to be suppressed, depending on if temperatures are rising into permissive temperature ranges (spring) or falling into non-permissive temperature ranges (fall; Rijkers 1982; Kennedy-Stoskopf 1993).

Only certain elements of the fish immune response are sensitive to temperature. Primary antibody responses are slower at low temperatures, but the magnitude and duration of the responses do not differ from the responses at higher temperatures (Kennedy-Stoskopf 1993). Normal secondary antibody responses may be elicited at low temperatures if the primary immunization is carried out at permissive temperatures (Avtalion 1969). Allograft rejection, and

helper T cell functions are effected by non-permissive temperatures, but memory T cell, B cell and accessory cell functions are not (Bly and Clem 1991). Antigen processing and presentation are also not effected by non-permissive temperatures (Vallejo, et al. 1992b).

Research indicates the temperature induced immunosuppression is not due to the induction of tolerance, and does not involve T suppressor cells or suppressor factors (Bly and Clem 1992). Furthermore, channel catfish T cells can be activated to proliferate at non-permissive temperatures *in vitro* by adding a combination of phorbol ester and calcium ionophore (Ellsaesser and Clem 1988; Lin, et al. 1992). This indicates that the effect of low temperature on T cell activation is an early event, occurring prior to protein kinase C activation (Bly and Clem 1992).

The temperature induced immunosuppression is thought to be due to a loss of membrane fluidity of the helper T cells and early blockade in the synthesis of T-cell-derived growth factors (Tizard 1992; Vallejo, et al. 1992b). The immunosuppressive effects of non-permissive temperatures can be overcome by acclimation. This allows time for homeoviscous adaptations to occur, which result in recovery of the membrane fluidity of the helper T cells (Ellis 1982; Bly and Clem 1991). The homeoviscous adaptation is due to changes which occur in plasma membrane fatty acid composition, with oleic acid (18:1) increasing and stearic acid (18:0) decreasing. The T and B cells are differentially susceptible to the effects of fatty acids and it was found that mitogen stimulated B cells could readily desaturate stearic acid to oleic acid, but T cells could not. Furthermore, it has been shown that the addition of oleic acid rescues the immune response of mitogen stimulated T cells at non-permissive temperatures *in vitro* (Bly and Clem 1992).

The syndrome "winter kill" occurs in catfish aquaculture ponds during the winter months when temperatures can drop rapidly. The syndrome is characterized by an invasion of opportunistic parasites and fungi similar to immunodeficiency syndromes seen in higher vertebrates. To mimic winter conditions in commercial ponds, channel catfish were exposed to "ultra-low", but physiologically relevant temperatures. The exposure resulted in suppression of both B and T cell functions for a period of 3-5 weeks. The response was found to be different than that of a typical stress-induced immunosuppressive response (Bly and Clem 1991).

The immunosuppressive effects of non-permissive temperatures on specific immune responses may be offset by increases in nonspecific immune responses. Le Morvan et al (1996) recently demonstrated that abrupt decreases in water temperature to non-permissive ranges resulted in increased NCC activity in carp compared to activity in NCC at permissive temperatures. They suggested that exposure of fish to non-permissive environmental temperatures does not result in full immunosuppression, but rather specific immunosuppression and nonspecific immunostimulation.

Stress

Stress is known to have physiological effects on fish which can be divided into three phases of response termed primary, secondary and tertiary. The primary effect of stress is characterized by elevations in plasma levels of corticosteroids and catecholamines of the neuroendocrine system (Mazeaud, et al. 1977). These endocrine responses bring about the metabolic and osmotic disturbances referred to as secondary effects, indicated by changes in plasma glucose, lactate and free fatty acid (Mazeaud, et al. 1977; Mazeaud and Mazeaud 1981). The tertiary effects include changes in behavior, decreased growth rate, and increased susceptibility to disease (Wedemeyer and McLeay 1981).

Factors known to induce a stress response in fish include osmotic and ionic changes, pollutants, anesthetics, crowding, handling, grading and hauling, as well as other aquaculture practices (Eddy 1981; Flos, et al. 1988). The primary response to stress occurs quickly and is of relatively short duration compared to the secondary and tertiary responses (Mazeaud and Mazeaud 1981; Schreck 1981). The primary responses to multiple acute handling stresses have been shown to be cumulative and consist of step-wise increases in plasma cortisol and glucose concentrations (Barton, et al. 1986; Flos, et al. 1988).

Corticosteroids can inhibit leucocyte migration, lymphocyte effector mechanisms, and inflammation in fish, as well as inhibiting interleukin production (Kennedy-Stoskopf 1993). Cortisol implants in juvenile coho salmon resulted in increased plasma cortisol levels, decreased levels of antibody secreting cells and decreased disease resistance (Maule, et al. 1987). Physiological concentrations of corticosteroids associated with stress and immunosuppression

in vivo can suppress the *in vitro* antibody response of coho salmon lymphocytes (Maule, et al. 1987). This suppression can be overcome by addition of conditioned media to the cortisol-suppressed cultures. The conditioned media is thought to contain a putative interleukin produced by macrophages (Kaattari and Tripp 1987).

Pollutants, Heavy Metals and Therapeutics

Relationships have been found to exist between the level of water pollution and the occurrence and frequency of diseases in the resident fish populations (Brown, et al. 1979; Overall, et al. 1991). In addition to the direct toxic effects of pollutants, the unhealthy environments of polluted sites are thought to evoke a stress response in fish which enhances disease susceptibility by suppressing the immune response (Brown, et al. 1979; Bennett and Wolke 1987a). Industrial discharges, city effluents, farm run-offs and other contaminants of human origin are sources of pollution in aquatic environments (Rougier, et al. 1994). The most common water pollutants include hydrocarbons, pesticides, herbicides and heavy metals. Responses to these agents range from suppression to enhancement of immune responses and appear to vary depending on the fish species and dosage.

In a field study, Arkoosh et al (1991) examined primary and secondary humoral immune responses of juvenile chinook salmon from an estuary contaminated with polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs). They found immune dysfunction manifested as a suppressed secondary plaque-forming-cell (PFC) response of anterior kidney leucocytes to TD and TI antigens. A follow-up laboratory study demonstrated these same immunosuppressive effects in juvenile chinook salmon injected with PAHs or PCBs (Arkoosh, et al. 1994). In contrast, the PFC response of channel catfish increased in fish injected with a low dosage of PCBs and was unaffected at higher doses (Rice and Schlenk 1995).

Macrophage chemotactic and phagocytic responses were decreased in spot (*Leiostomus xanthurus*) and hogchokers collected from a river with high levels of PAHs (Weeks and Warinner 1986). However, macrophage chemiluminescent (CL) responses, which measure the production of oxygen free-radicals, were increased in mummichog (*Fundulus heteroclitus*) from another

river heavily contaminated with PAHs. Macrophage CL responses returned to normal levels when mummichog were transferred to clean water, and fish transferred from clean water contaminated river water began to exhibit increased CL responses (Kelly-Reay and Weeks-Perkins 1994). Non-specific cytotoxic cell activity was reduced only at the highest doses in channel catfish injected with PCBs (Rice and Schlenk 1995).

The effects of herbicides and pesticides on the fish immune system have been examined in several laboratory studies. Dioxin, a by-product of the production of some herbicides, had no effect on phagocytosis or humoral and cellular immunity in rainbow trout (Kennedy-Stoskopf 1993). The pesticide malathion was found to decrease serum agglutination titers (SAT) in chronically exposed channel catfish (Plumb and Areechon 1990), and Nile tilapia (*Oreochromis niloticus*) exposed to low levels of the organophosphate pesticide chlorpyrifos had significantly lower total pronephros cell counts and depressed macrophage phagocytic ability compared to untreated controls (Holladay, et al. 1996). Sublethal exposure of the organochlorine pesticide endrin had no effect on the phagocytic ability of rainbow trout peritoneal macrophages, but significantly reduced MIF, PFC and SAT responses (Bennett and Wolke 1987a). Serum cortisol levels were also significantly higher. In a follow-up study to determine if the immunosuppression was due to the endrin, increased cortisol levels or both, the MIF response was completely restored and the PFC and SAT responses were partially restored in endrin exposed fish fed metyrapone, a corticoid synthesis blocker (Bennett and Wolke 1987b). These results suggested the immune suppression was not a direct effect of the endrin, but an effect of the increased cortisol which resulted from the stress response evoked by the endrin exposure.

A number of heavy metals immunotoxic in mammals also alter immune functions in fish. These alterations may result in increased host susceptibility to infectious and malignant diseases in fish living in environments polluted with heavy metals. The metals whose effects have been studied in fish include aluminum (Al^{3+}), cadmium (Cd^{2+}), chromium (Cr^{6+}), copper (Cu^{2+}), lead (Pb^{2+}), manganese (Mn^{2+}), nickel (Ni^{2+}), tin (Sn^{2+}) and zinc (Zn^{2+} ; Zelikoff 1993).

Anemia is a common toxic effect of exposure to heavy metals in fish, but the effects of heavy metals on leucocytes and nonspecific immune responses are variable (Kennedy-Stoskopf

1993). *In vitro* assays of immune function are altered by many heavy metals. The metals Al^{3+} , Cu^{2+} and Ni^{2+} have been shown to decrease macrophage responses and the metals Cd^{2+} , Mn^{2+} , Sn^{2+} and Zn^{2+} have been shown to enhance macrophage responses (Elsasser, et al. 1986; Zelikoff 1993; Rougier, et al. 1994). Leucocyte function is effected by Cu^{2+} , which results in a reduced PFC response and NCC response, Zn^{2+} , which has mitogenic effects, and Mn^{2+} , which enhances mitogen induced proliferation and increases NCC cytotoxicity (Zelikoff 1993; Rougier, et al. 1994). *In vivo* assays of immune function altered by heavy metals include reports of both increases and decreases in antibody titers with Cd^{2+} and decreases in humoral responses with Cr^{6+} , Cu^{2+} , Pb^{2+} , Ni^{2+} and Zn^{2+} (Zelikoff 1993).

Therapeutic agents may also be immunosuppressive to fish. Oxytetracycline has been shown to be immunosuppressive in a number of fish species (Anderson, et al. 1984). Oxytetracycline delays the primary antibody response, and decreases the number of cells in the PFC response (Kennedy-Stoskopf 1993). Tetracycline and oxytetracycline have been shown to cause dose-dependent suppression of the kidney macrophage CL response in rainbow trout (Wishkovsky, et al. 1987).

Nutrition

Nutrition is known to influence specific and nonspecific immune responses in fish. Micronutrients known to affect disease resistance in fish include vitamins A, B₆, C, and E, as well as fatty acids (Blazer 1992). Vitamin A (retinol) is a fat-soluble vitamin that, along with carotenoids, is known to affect a number of diseases resistance mechanisms in homeotherms. Dietary vitamin A is important for maintaining the integrity of epithelial and mucosal surfaces, and for the production of mucous secretions (Blazer 1992). The effects of vitamin A on disease resistance in fish has not been well studied, but there are indications dietary vitamin A can reduce salmon mortalities to the parasite *Ichthyophthirius multifiliis*, and that it can enhance catfish NCC activity against parasites (Blazer 1992).

Vitamin B₆ (pyridoxine) is a water soluble vitamin important in homeotherm immune responses. Animals deficient in dietary pyridoxine have been shown to be inhibited in their ability

to incorporate amino acids into tissue proteins and their ability to synthesize DNA and RNA, particularly messenger RNA (Blazer 1992). Both vaccinated and non-vaccinated chinook salmon had higher survival against *Vibrio anguillarum* when they were fed pyridoxine together with a high protein diet, but not with a low protein diet (Hardy, et al. 1979). The pyridoxine appeared to be influencing nonspecific resistance mechanisms since there was no difference in antibody titers and mortality was reduced in non-vaccinated fish at higher pyridoxine levels (Hardy, et al. 1979; Blazer 1992).

Vitamin C (ascorbic acid) is another water soluble vitamin that has been implicated in disease resistance in homeotherms. It is a biological reducing agent for hydrogen transport involved in a number of enzyme reactions, and it is an essential factor in the regulation of cortisol synthesis. It is also necessary for maintaining the epithelial barrier and for wound healing (Blazer 1992). Vitamin C is the best studied vitamin in fish nutrition. A number of studies using bacterial pathogens in fish suggest dietary levels of ascorbic acid above those required for growth give some level of protection (Blazer 1992). In Atlantic salmon, vitamin C deficiencies had no effect on serum protein levels, differential leucocyte counts, macrophage respiratory burst activity, phagocytosis or antibody production. However, mortality due to challenge with *Aeromonas salmonicida* was significantly higher in vitamin C deficient fish, and serum complement activity was significantly reduced (Hardie, et al. 1991). In rainbow trout with ascorbic acid deficiencies, peritoneal macrophages were found to have a greatly reduced ability to engulf latex beads (Blazer 1991).

Vitamin E (α -tocopherol) is a fat soluble vitamin. It is a component of cell membranes where it functions as the primary antioxidant, protecting unsaturated fatty acids against oxidative damage (Wise, et al. 1993). Vitamin E is very important in fish nutrition because fish maintain membrane fluidity at low temperatures by having a high percentage of long chained, highly unsaturated fatty acids incorporated into their cell membranes (Blazer 1992). Humoral and cellular specific and nonspecific immune responses were found to be significantly reduced in vitamin E deficient rainbow trout (Blazer and Wolke 1984). In contrast, only increased mortality to *A. salmonicida* infections and decreased complement activity were seen in vitamin E depleted

Atlantic salmon (Hardie, et al. 1990). Channel catfish fed supplemental levels of vitamin E were found to have an increased antioxidant capacity of red blood cells and enhanced macrophage phagocytosis, but specific humoral antibody responses were not effected (Wise, et al. 1993).

Dietary lipids, especially certain fatty acids, are important modulators of the immune responses of homeotherms. Dietary lipids also strongly influence fatty acid composition of cell membranes, which in turn affects membrane fluidity and permeability (Blazer 1992). Dietary lipids, particularly n-3 long chain highly unsaturated fatty acids, were found to effect the intracellular killing of *E. ictaluri* by channel catfish kidney macrophages (Sheldon and Blazer 1991).

THE CATFISH IMMUNE RESPONSE TO ESC

Catfish that recover from natural *E. ictaluri* infections are generally assumed to be resistant to future infections (Klesius and Horst 1991). The mechanisms of protective immunity have not been well studied, though both humoral and cellular mechanisms appear to be important. In particular, the combination of the components of immune serum together with phagocytic cells appears to play an important role in the catfish immune response to *E. ictaluri*. The role of T cells in the protective immune response has not been investigated, although their importance has been suggested due to observational evidence which suggests that *E. ictaluri* may be a facultative intracellular bacterium (Thune, et al. 1997b). Investigations of the humoral and phagocytic aspects of the catfish immune response against *E. ictaluri* are reviewed here.

Bactericidal Activity of Serum

Normal mammalian serum was found to have no bactericidal effects on viable *E. ictaluri* cells (Janda, et al. 1991a). Ourth and Bachinski (1987) found only 8% bactericidal activity of normal catfish serum against *E. ictaluri*, which they correlated with the relatively high amount of sialic acid on the surface of the bacteria. Since sialic acid is known to inhibit the alternate complement pathway, they suggested this was the mechanism of resistance (Ourth and Bachinski 1987). The literature appears to be lacking any studies on the bactericidal activity of serum from immune catfish against *E. ictaluri*. With immune serum, antibodies against *E. ictaluri*

that bind to the cell surface could initiate the classic complement pathway. For Gram negative bacteria such as *E. ictaluri*, destruction by complement is a "two-hit" process. In the first step the complement membrane attack complex disrupts the outer membrane and exposes the inner membrane. The second step is the breakdown of the inner peptidoglycan membrane by lysozyme (Tizard 1992). Complement activation by the classic pathway can also result in cell-mediated chemotaxis of granulocytes and phagocytic cells, complement mediated opsonization and complement mediated inflammation (Tizard 1992). Whether or not the classic pathway of complement activation is important in the immune response against *E. ictaluri* still needs to be determined.

Humoral Antibody Responses

Many studies have examined the catfish immune response to a variety of ESC vaccine preparations and delivery methods, but few studies have examined the immune responses of catfish that have recovered from natural infections. The antibody responses of catfish to vaccination are reviewed later with ESC vaccines. *Edwardsiella ictaluri* is highly immunogenic (Plumb 1984). Infection by *E. ictaluri* induces specific antibodies detectable by agglutination, ELISA, FAT and Western blotting. The role of specific antibodies in protection has not been determined definitively.

Antibodies and Protective Immunity

Vinitnantharat and Plumb (1993) showed that individual catfish in populations that survived natural *E. ictaluri* infections differ in their antibody responses, and these differences correlate with protective immunity against ESC. In this study, catfish that survived a natural *E. ictaluri* infection were grouped by serum antibody levels into no, low, medium and high responders with agglutination titers of 0, 128, 256-512 and ≥ 1024 respectively. When these fish were challenged with *E. ictaluri* by intraperitoneal injection, there was a very strong relationship ($R=0.99$) between the degree of protection and the antibody titer (Vinitnantharat and Plumb 1993). An agglutination titer over 256 was necessary to ensure detectable protection against injection challenge. Since this study used an unnatural challenge route (intraperitoneal injection)

any mucosal immune responses, which may be important in protective immunity to ESC, were bypassed.

Antibody Specificity

The specificity of channel catfish antibodies against *E. ictaluri* has been evaluated in sera collected from naturally (Chen and Light 1994) and laboratory infected fish (Tyler and Klesius 1994c). Microtiter agglutination assays showed *E. ictaluri* antibodies in the sera of naturally infected fish had no cross-reactivity with nine other species of bacteria (including *E. coli*, ATCC 25922) found in the catfish intestine and fish ponds (Chen and Light 1994). Sera from catfish that survived a laboratory challenge (presumably by immersion) with virulent *E. ictaluri* were evaluated for cross-reactivity against *E. coli* (Tyler and Klesius 1994c). An ELISA assay with whole cells of either *E. ictaluri* or *E. coli* 0111:B4 (a rough mutant strain) as the antigen showed a high amount of cross-reactivity with the *E. coli* (Tyler and Klesius 1994c). Some, but not all, of the reactivity to both the *E. ictaluri* and *E. coli* cells was absorbed out with purified LPS from a third bacterial species, *Salmonella typhimurium* TV119 (a rough mutant strain) (Tyler and Klesius 1994c). From these results, the authors concluded that natural exposure to *E. ictaluri* induces both specific and cross-reactive humoral antibodies, and much of the cross-reactivity is to the homologous core region of the LPS layer of the cell wall of gram negative bacteria (Tyler and Klesius 1994c).

Both the agglutination (Chen and Light 1994) and ELISA (Tyler and Klesius 1994c) assays used whole cells, which limited detection to cross-reactive antibodies that recognized cell surface antigens. The ELISA assay is more sensitive than microtiter agglutination, which probably explains the difference in the results of the two studies. The importance of cross-reactive antibodies is discussed further in the review of ESC vaccines.

Antigens of *E. ictaluri*

Catfish that have recovered from natural *E. ictaluri* infections produce antibodies against a number of *E. ictaluri* proteins as well as LPS. Baldwin et al (1997) determined the antigenicity

of *E. ictaluri* proteins in whole cell lysates (WCLs) and enriched bacterial fractions using SDS-PAGE and Western blotting. Antigenic proteins were identified in Western blots using pooled convalescent catfish serum collected from pond-raised catfish surviving natural *E. ictaluri* infections. A total of 38 proteins were identified in WCLs, 14 of which were antigenic. Using enriched bacterial fractions in conjunction with WCLs they determined six of the 14 antigenic proteins were outer membrane proteins with apparent molecular weights of 56, 44, 39, 35, 18 and 16 KD, and one was a flagellar protein (37 KD). The locations of the remaining seven antigenic proteins (70, 65, 33, 32, 29, 22 and 14 KD) were unknown. Two of the 14 WCL proteins (39 and 37 KD) and LPS were recognized strongly by the convalescent catfish sera (Baldwin, et al. 1997).

Humoral Factors and the Phagocytic Immune Response

Humoral factors in both normal and immune catfish sera appear to be important in the immune response to *E. ictaluri*, particularly as opsonins for phagocytic cells. Since most phagocytic studies are done using laboratory held catfish, the immune fish are obtained through vaccination, rather than being survivors of natural infections. The results of these studies may be affected by differences in the vaccination procedures.

Macrophages

Specific opsonins and heat-labile serum factors were both found to have an effect on the CL response of catfish PBL mononuclear phagocytes following phagocytosis of *E. ictaluri* cells (Scott, et al. 1985). Opsonization of bacteria with sera from immune catfish resulted in a peak CL response about ten times greater than opsonization of bacteria with serum from normal catfish, or with non-opsonized bacteria (Scott, et al. 1985). The fish in this study were vaccinated with formalin killed *E. ictaluri* whole cells in Freund's complete adjuvant (FCA) given in three IP injections one week apart. Opsonization of bacteria with immune serum absorbed to remove specific antibodies against *E. ictaluri* resulted in a more than 60% decrease in the CL response, as well as an increase in the time to peak response from 3 minutes to 8 minutes. Heat inactivation of the immune serum before opsonization resulted in a 40% decrease in CL response and an increase in time to peak response from 3 minutes to 8 minutes. In both cases, the

addition of normal serum did not change the CL response, but restored the peak response time back to 3 minutes (Scott, et al. 1985). The CL response of mononuclear phagocytes from immune catfish was consistently significantly higher than responses from normal phagocytes. The authors suggested the enhanced CL response, though mediated by serum factors, was due to a general activation of the phagocyte (Scott, et al. 1985).

The CL assay is an indirect measure of phagocyte oxygen-independent bactericidal activity, which requires myeloperoxidase. The assay is used as an indicator of both phagocytosis and intracellular killing (Blazer 1991), though it has been shown experimentally that a substantial percentage of the CL response is an extracellular event (Briheim, et al. 1984). The CL response of human PMN leukocytes has been shown to be a two-peak response (Briheim, et al. 1984). The first peak, at 2 minutes, is an extracellular event and the second peak, at 8 minutes, is an intracellular event. The longer time to the second peak appears to be due to the diffusion rate of luminol into the cell, and the height of the second peak is related to the concentration of luminol used in the assay (Briheim, et al. 1984). With this in mind, it is possible the time shifts seen in the study by Scott et al (1985) are due to shifts in the CL response from primarily extracellular to primarily intracellular events.

In a study by Blazer and colleagues (Sheldon and Blazer 1991), macrophages had significantly higher bactericidal activity against *E. ictaluri* cells opsonized with autologous serum from immune catfish compared to cells opsonized with autologous serum from normal catfish. There was no difference in phagocytosis between the treatments. The fish were vaccinated with a single IP injection of formalin killed *E. ictaluri* whole cells without adjuvant. Bactericidal activity and phagocytosis were measured with a differential fluorescent staining procedure (Sheldon and Blazer 1991). The fluorescent stain technique is a direct measure of bactericidal activity (Blazer 1991). The substantially enhanced bactericidal activity of the immune catfish was thought to be due to activation of the macrophages (Sheldon and Blazer 1991). Since there was no difference in phagocytosis between normal and immune fish, the authors suggested the enhanced bactericidal activity of immunized fish was not due to opsonization by specific antibody, but

rather the activation of macrophage killing, indicating macrophage killing activation may be an important protective immune response against *E. ictaluri* (Sheldon and Blazer 1991).

In a subsequent study by Blazer and colleagues (Wise, et al. 1993) however, macrophages from immune fish were shown to have a significantly higher ability to phagocytized *E. ictaluri* cells opsonized with homologous serum compared to macrophages from non-immune fish, but only when levels of dietary vitamin E were adequate. Bactericidal activity was not evaluated. There was no significant difference in macrophage phagocytosis between immune and non-immune catfish fed diets deficient in vitamin E. The fish were vaccinated by a 30 second immersion in a bath of formalin-killed *E. ictaluri*, followed with an oral booster on days 120, 121 and 122 (Wise, et al. 1993). Assuming the dietary levels of vitamin E were adequate in the first study (Sheldon and Blazer 1991), the difference in the macrophage phagocytosis results of the two studies may have been due to the differing routes of vaccination that were used.

Bactericidal activity against *E. ictaluri* by squalene-elicited peritoneal macrophages was significantly higher in catfish vaccinated with a controlled-live exposure to *E. ictaluri* than in fish vaccinated by immersion or oral routes with commercial bacterins (Shoemaker and Klesius 1997). Opsonization of *E. ictaluri* with immune sera further increased the bactericidal activity of the macrophages from controlled-live vaccinates (Shoemaker, et al. 1997). Controlled-live vaccination of catfish with *E. ictaluri* entailed immersion exposure to a low level of bacteria. This method of vaccination resulted in the production of specific antibodies and increased macrophage bactericidal activity. Subsequent challenge experiments showed the fish were protected against ESC. The results of the study suggested both specific antibodies and increased bactericidal activity were associated with greater survival against ESC (Shoemaker and Klesius 1997).

Neutrophils

Neutrophils from normal catfish were shown to phagocytize *E. ictaluri* cells opsonized with autologous serum (Finco-Kent and Thune 1987). No bactericidal activity was measured using uptake of ^3H -uridine as an indicator (Finco-Kent and Thune 1987). The incorporation of ^3H -uridine measures RNA synthesis, which is an indirect measure of oxygen-dependent phagocyte respiratory burst activity (Blazer 1991).

In another study, neutrophils from normal catfish had significantly higher phagocytosis of *E. ictaluri* when cells were opsonized with autologous serum (63.3% phagocytosis) compared to when cells were opsonized with heat inactivated autologous serum (8.6% phagocytosis; Ainsworth and Dexiang 1990). In addition, at 60 minutes significant killing or stasis of *E. ictaluri* was seen in bacterial cells opsonized with normal serum, but not in cells opsonized with heat inactivated serum (Ainsworth and Dexiang 1990). Killing was evaluated by plating aliquots of bacteria which had been incubated with neutrophils for 60 minutes (Ainsworth and Dexiang 1990). This type of bactericidal assay is a direct measure of phagocyte intracellular killing (Blazer 1991). In a subsequent study however, using longer incubation times of 180 minutes, the bactericidal assays did not demonstrate intracellular killing of *E. ictaluri* by catfish neutrophils (Waterstrat, et al. 1991). Light and electron microscopy showed the *E. ictaluri* cells were in well-defined phagocytic vacuoles within the neutrophils. The catfish neutrophils did mount a CL response to *E. ictaluri* opsonized with immune serum twice that of non-opsonized *E. ictaluri* or *E. ictaluri* opsonized with non-immune serum (Waterstrat, et al. 1991). In addition, extracellular bactericidal activity was observed which was due to either serum components, extracellular products liberated from the neutrophil, or a combination of both cellular and humoral factors (Waterstrat, et al. 1991). The CL responses that were measured were thought to be due to extracellular events because they were enhanced by immune sera and extracellular bactericidal activity was observed. The authors suggested that although *E. ictaluri*'s apparent ability to survive in the neutrophil could serve as a means of disseminating the bacteria through-out the

host, other phenomenon could be operating, particularly in regard to the importance of extra-cellular killing by the neutrophils (Waterstrat, et al. 1991).

VACCINATION STRATEGIES IN FISH

Rationale

Although epizootics are known to occur in wild fish populations, the major problems caused by infectious agents in fish occur in farmed fish. Like other intensely farmed species, the high stocking densities necessary for the financial success of an aquaculture facility predispose fish to diseases due to pathogenic organisms, often as a direct result of stressors inherent in this type of farming (Ward 1982; Newman 1993). Therapeutic methods are available for treating fish diseases, but they have limitations. Only two antibiotics are approved for use in catfish in the United States and they are used extensively. There are concerns that overuse or misuse of these antibiotics will lead to antibiotic resistance in the target bacteria (Anderson 1992).

Chemotherapy with antibiotics is usually not started until the causative agent of the disease outbreak has been confirmed, by which time a significant percentage of the fish might be infected (Ward 1982). A proportion of the infected fish cannot be treated because they stop feeding and do not receive any of the antibiotic, which is administered in the feed. These untreated fish will usually die. The cost of treatment then includes not only the cost of the medicated feed, but also the cost of the mortalities. For these reasons, alternative strategies of disease control in fish have been sought (Ward 1982).

Vaccination has been used successfully and is well-established in most areas of animal husbandry. Unlike antibiotics, which are used therapeutically, vaccines are used prophylactically (Ward 1982). Since prevention of a disease is much more desirable than treatment of a disease once it has occurred, vaccination is usually the method of choice for controlling infectious diseases. An ideal vaccine is one that is relatively inexpensive to produce, easy to administer, safe and effective (Kennedy-Stoskopf 1993). Despite a lack of knowledge of the mechanisms involved in the protective immune response against the pathogens, vaccination has been used successfully to control several diseases in fish (Ward 1982; Newman 1993).

Vaccine Delivery Methods

Three methods, with a number of variations, are currently used to deliver vaccines to fish. The methods vary in their effectiveness, and each has advantages and disadvantages.

Injection

Injected vaccines evoke the highest levels of protection in fish (Newman 1993). In addition, injection requires less antigen than other delivery methods and allows the use of adjuvants which enhance the magnitude of the immune response (Ellis 1988a). Vaccines can be injected intraperitoneally (IP), intramuscularly (IM), intravenously (IV) or subcutaneously (SC). Intramuscular injection elicits a higher antibody response and better memory response than IP and IV respectively (Ellis 1988b). However, intramuscular injection on the dorsal surface of the fish is rarely used on the commercial scale because it may cause unsightly scarring and some of the vaccine may leak from the injection site before absorption has occurred (Home and Ellis 1988).

The injection method of vaccine delivery in fish does have several disadvantages. It requires more time and people to inject fewer fish than other delivery methods. Injection also requires that the fish be anesthetized and individually handled, both of which are stressful and can cause mortalities. In addition, injection can be hazardous to the persons doing the injecting (Ellis 1988a; Newman 1993). Semi-automated vaccination devices have been developed that eliminate accidental injection of the worker, minimize stress on the fish, and can immunize up to 4000 fish per hour. These devices may help make injection the preferred method of vaccination, especially in larger fish, broodstock, or more commercially valuable species such as salmonids (Newman 1993).

Immersion

In the early 1970's, Amend and colleagues discovered that fish had the ability to take up antigenic material from an aqueous environment, and this could be used as a method of vaccination (Amend and Fender 1976; Antipa and Amend 1977). Initially the method was a two-step procedure that began with immersion in a hyperosmotic solution, followed by immersion in

a suspension of antigen. This procedure was called hyperosmotic immersion (HI; Antipa and Amend 1977). The hyperosmotic step induced severe osmotic shock which was thought to improve antigen uptake, but it was very stressful on the fish (Ellis 1988a). It was subsequently determined that the osmotic shock was not necessary, and vaccination by direct immersion (DI) in the antigen suspension was equally successful (Antipa, et al. 1980).

Vaccination by DI induces a high level of protection, and is the most widely used method of vaccination in fish. The method is not as stressful on fish as injection and larger numbers of fish can be vaccinated in a shorter amount of time (Newman 1993). The method is simple and rapid, only a few seconds of exposure to the vaccines are necessary and immersion vaccination machines are now available (Ellis 1988a). The disadvantages of the DI method are that it is labor intensive, fish must be handled, and it is not economical for use on larger fish. In addition, a method of delivering adjuvants in conjunction with the vaccine has not yet been developed (Newman 1993).

A variation of the DI method is the bath or flush method of vaccination. This involves pouring the vaccine into the holding tanks to avoid handling stress (Ellis 1988a). Bath immersion (BI) allows *in situ* immunization in hatchery troughs, holding tanks, transport vessels and net pens (Newman 1993). The disadvantages of BI compared to DI are that more vaccine and longer exposure times are required. This necessitates aerating the holding tanks and closely monitoring the fish for signs of stress (Ellis 1988a).

Another variation of the DI method is spray vaccination. In this method, fish are sprayed with vaccine, rather than being immersed in it. The antigen is sprayed under pressure on to the fish as they are propelled along a shallow channel (Ward 1982). The method is effective as long as the fish are exposed to the vaccine for at least two minutes (Newman 1993). This method is the more economical than DI because three to 10 times the poundage of fish per unit volume can be vaccinated. This also makes it more economical to immunize larger fish (Newman 1993). The disadvantages of spray vaccination are that the fish must be handled, it is labor intensive, and specialized machinery is required (Newman 1993).

Oral

With the oral route of immunization, fish are fed inactivated bacteria in a paste or liquid suspension that has been coated onto or milled into the feed (Newman 1993). This is the most useful method of vaccination because it is non-stressful, it can be used on any size fish, and it does not require extra labor. The disadvantages are that it takes more antigen to obtain effective immunity, and the immune response is lower and of shorter duration than with other methods (Ward 1982).

It is thought that the lower immunogenicity of the oral vaccines is due to denaturation or inactivation of the antigens in the high acid environment of the stomach. An oral enteric-coated *V. anguillarum* vaccine was developed by coating lyophilized bacteria onto dextrose sugar beads, then coating with Eudragit L-30D to serve as enteric protection against stomach acid (Wong, et al. 1992). This vaccine was designed to withstand the high acid environment of the stomach and be released rapidly in the intestine where both systemic and local mucosal immunity could be stimulated. The study showed the efficacy of oral vaccines can be improved if enteric-coating is used to protect the antigen from stomach acids (Wong, et al. 1992).

Vaccine Types

There are numerous types of vaccines used in mammalian medicine, including use in humans. The types of vaccines shown to have potential in fish will be discussed. They include inactivated whole or disrupted bacteria, attenuated live vaccines, subunit and synthetic peptide vaccines, vaccine vectors, and DNA vaccines. The use of adjuvants, immunostimulants and vaccine carriers with fish vaccines is also discussed.

Inactivated

Inactivated bacterial vaccines contain bacterial cells killed by heat or chemical treatment. Ideally inactivation should not alter the antigens responsible for stimulating protective immunity. Heat inactivation is usually not satisfactory because it causes extensive protein denaturation or lipid oxidation. Formaldehyde is often used because it confers structural rigidity by cross-linking

amino and amide groups in proteins, and hydrogen-bonded amino groups in the purine and pyrimidine bases of nucleic acids (Tizard 1992).

Inactivated bacterial vaccines may consist of formalin-killed whole cells, or formalin-killed cells disrupted by freeze-thawing, french press or sonication. The advantages of inactivated vaccines are that they are safe with respect to residual virulence and relatively easy to store. The disadvantages are they are poorer immunogens than live bacteria, and the adjuvants used with them to compensate for lower immunogenicity may result in severe local reactions or hypersensitivity responses (Tizard 1992).

Inactivated bacterial vaccines are the type of vaccine used most often, and with the most success in controlling fish diseases. Inactivated vaccines for controlling vibriosis, caused by *Vibrio anguillarum*, *V. ordalii*, and *V. salmonicida*, and enteric redmouth disease, caused by *Yersinia ruckeri*, have all been very successful in controlling disease and have significantly improved profitability (Newman 1993). These commercial vaccines were easy to develop because they were successful without any understanding of the nature of the pathogenic mechanisms of the bacteria or the immune response of the host (Newman 1993). There is little incentive to improve these vaccines, which are all formalin-killed whole cell suspensions that are very easy and inexpensive to prepare. Sonicated suspensions have been shown to offer higher levels of protection against these bacteria, but they are more costly to produce (Newman 1993).

Unfortunately, inactivated bacterial vaccines have been less successful with other fish pathogens. Commercial vaccines are available against furunculosis, caused by *Aeromonas salmonicida*, but their performance has not been as good as those of the vibrios and ERM (Newman 1993). Commercial vaccines have also been licenced against ESC, but their success was equivocal, and at present they are not being produced (Thune, et al. 1997b). The success of inactivated *E. ictaluri* vaccines in catfish is reviewed in the section on ESC vaccines.

Attenuated

Attenuated vaccines contain viable organisms with reduced virulence. The virulence has been attenuated to the point where the bacteria or viruses can infect the host, but they can no

longer cause disease. Attenuation can be achieved by culturing the bacteria under adverse conditions such as in the presence of heat, chemicals or nutrient shortage (Tizard 1992). Viruses can be attenuated by repeated passages in tissue culture or unnatural hosts (Kennedy-Stoskopf 1993). One advantage of attenuated vaccines are that they are better immunogens than inactivated vaccines. Generally, viable bacteria are better at stimulating cell-mediated immune responses than inactivated bacteria. The greater efficacy of live vaccines over inactivated may also be due a better distribution of live bacteria within the host, as well as the antigenic and biochemical changes that occur as a result of the inactivation process (Tizard 1992). Attenuated vaccines require fewer inoculating doses than inactivated vaccines, and they elicit higher antibody titers (Kennedy-Stoskopf 1993). Attenuated vaccines also do not require adjuvants, probably because they are able to induce cytokine production in the host better than inactivated vaccines (Tizard 1992). The disadvantages of attenuated vaccines include residual virulence and the potential for reverting back to full virulence. Live vaccines are also more susceptible to contamination by other organisms, and they require more care in preparation, storage and handling to maintain viability (Tizard 1992).

A refinement of the attenuation process uses genetic engineering to deliberately modify genes so the organism becomes permanently attenuated (Tizard 1992). The advantages of this type of attenuation are that the mutations can be defined, and they are less likely to revert. In addition, the genetically attenuated bacteria can be tagged with phenotypic markers, such as antibiotic resistance, so they can be distinguished from the wild-type bacterium or virus.

Attenuated vaccines for fish pathogens are not commercially available, though several have been tested experimentally. Three attenuated strains *A. salmonicida* have been evaluated as vaccines against furunculosis in salmonids. A virulent strain of *A. salmonicida*, attenuated by repeated laboratory passages was found to induce significant protection in brook trout (*Salvelinus fontinalis*) and Atlantic salmon vaccinated by IP injection or immersion (Cipriano and Starliper 1982). The nature of the attenuation was not defined, but may have been due to loss of the external A-layer (Vaughan, et al. 1993). Two other attenuated strains of *A. salmonicida*, a slow

growing strain that had lost anaerobic metabolism and a faster growing partial revertant with reduced ability to metabolize carbon sources, were both found to be protective in chinook salmon when administered by IP injection and immersion (Thomton, et al. 1991).

A genetically attenuated *A. salmonicida* strain has also been developed (Vaughan, et al. 1993). This strain is an auxotrophic, aromatic-dependent mutant with a kanomycin resistance gene inserted into the *aroA* gene. The mutation was found to be stable, and induced a higher level of protection in brown trout than any of the other attenuated *A. salmonicida* strains (Vaughan, et al. 1993). A pathogenic fish virus has also been genetically attenuated. Channel catfish virus (CCV; *Ictalurid herpesvirus 1*) was attenuated by constructing a recombinant thymidine kinase (TK) gene deletion mutant. The attenuated virus was shown to induce protective immunity against the wild-type CCV (Zhang and Hanson 1995). Recently, a genetically attenuated strain of *E. ictaluri* was developed and evaluated as a vaccine (Lawrence, et al. 1997). The recombinant adenine auxotrophic strain of *E. ictaluri*, with a deletion and Kanamycin resistance gene insertion in the *purA* gene, was shown to have potential as a vaccine against ESC. The results of this study will be discussed further in the section on ESC vaccines.

Subunit and Synthetic Peptides

Two other vaccine types are subunit and synthetic peptide vaccines. Both techniques require the identification of the antigens that evoke a protective immune response in the host. With subunit vaccines, the antigen of interest is purified from the bacteria or virus. This can be labor intensive, and the amount of antigen obtained may be limited. Another strategy uses the molecular cloning of genes from the pathogen encoding proteins important as virulence factors or protective antigens (Munn 1994). These subunit vaccines are produced in *E. coli*, yeast or insect cell cultures that can express large amounts of the protective antigens. The expressed protective proteins can then be used as vaccines either in cell lysates of the recombinant expression system or as purified proteins (Munn 1994).

In some cases, the development of a successful vaccine may require focusing on the immune responsiveness of the host to a few molecules out a mixture of potential antigens

(Vallejo, et al. 1992a). One reason for the limited success of vaccines containing killed bacterins may be that they are deficient in protective antigens (Munn 1994). The ability to produce antibodies to a mixture of antigens in a vaccine does not imply immune protection. In some cases, immunodominant antigens in a mixture may result in a strong antibody response to only a few proteins. The "lesser antigenic" molecules may actually be the protective antigens, but in killed bacterins they are in quantities too low to induce a response. This is supported by observations that exposure to purified lesser antigenic molecules can elicit a strong specific immune response (Vallejo, et al. 1992a).

An example of this phenomenon has been seen in fish vaccines to furunculosis extra-cellular products (ECP). These vaccines elicit a strong antibody response, but antibodies are only produced against four of 20 ECP (Vallejo, et al. 1992a). One of the 16 ECP that does not elicit an antibody response is the protease caseinase. However, salmon can be protected by passive immunization with rabbit anti-caseinase antibodies and salmon do produce antibodies against partially purified caseinase. This demonstrates that the development of a successful vaccine in some cases may require focusing on the immune responsiveness of fish to a few molecules out a mixture of potential antigens (Vallejo, et al. 1992a). Since OMP are viewed as being extremely important in pathogenicity, these are often the targeted proteins (Munn 1994).

An even further refinement of the subunit vaccine is the use of synthetic peptides. With this approach, the immunologically relevant parts of the antigen are identified by sequence analysis and epitope mapping (Munn 1994). If the nucleic acid sequence of the gene encoding the protective antigen is known, the protective epitopes can be predicted using computer programs that first translate the DNA sequence data into the amino acid sequence, then identify hydrophilic areas on the protein (Tizard 1992). Once identified, the protective epitopes can be synthesized and used as a vaccine. Since a number of genes encoding antigens from fish pathogens have been cloned into expression vectors and sequenced, this is a feasible approach to use in the development of fish vaccines (Munn 1994). The synthetic peptide vaccines are safer than other recombinant vaccines. Unfortunately, it is very expensive to synthesize peptides (Tizard 1992).

Another disadvantage of both the subunit and synthetic peptide vaccines is that very pure proteins are often poor immunogens. There are several ways to increase the immunogenicity of these vaccines. In the case of the subunit vaccines, one solution is to use the lysate of the recombinant host cells as a vaccine, rather than the purified protein. Over expression of the antigen by the recombinant cells enables the induction of an immune response, even in the presence of other immunodominant proteins. In addition, antigens in the recombinant host cell may also help to stimulate the immune response. Another approach is to incorporate the antigen into solid particles called immunostimulatory complexes (ISCOMs). These complexes are generated by mixing the antigen with a biocompatible detergent and the complex adjuvant Quil A (Rabinovich, et al. 1994). The immunogenicity of purified proteins can also be improved by conjugating them with carrier proteins (Ada 1993).

There are several approaches for improving the immunogenicity of synthetic peptides. One approach is to link both B and T cell epitopes in a single peptide. The mechanics of this type of approach still need to be optimized (Rabinovich, et al. 1994). Another approach is to use multiple antigen peptide systems (MAPS), which consist of selected T and B cell epitopes conjugated onto a polylysine core without a carrier protein (Rabinovich, et al. 1994). Although B and T cell epitopes have not yet been defined for any of the fish pathogens, the MAPS approach has been tested in fish and found to be a suitable delivery system (Riley, et al. 1996).

Vectors

Vaccine vectors use a non-pathogenic virus or bacteria to deliver protective antigens of a different pathogen to a host. Like subunit and peptide vaccines, the protective antigens of the pathogen have to be identified and cloned. The genes encoding the protective antigens are then inserted into a virus or bacteria that can over express the recombinant protein (Rabinovich, et al. 1994). Unlike the regular expression systems, these vectors are able to infect the host, but since they are attenuated they do not cause disease. If the vaccine vector is a virus, then the inserted foreign genes will be expressed in the context of MHC class I antigens, which will induce a cytotoxic T cell response. In mammals, *Vaccinia* virus is the vector most commonly used for

the expression of foreign genes. A number of *Salmonella* species have been evaluated for their potential to serve as bacterial vectors (Rabinovich, et al. 1994).

The attenuated recombinant strain of CCV described previously (Zhang and Hanson 1995), is able to express the proteins of foreign genes inserted in frame with the promoter of the deleted TK gene (Zhang and Hanson 1996). Catfish vaccinated with this CCV vector produced antibodies against the foreign gene product, indicating the foreign gene was expressed in the host, and the gene product was immunogenic. If protective antigens against other catfish pathogens, such as *E. ictaluri* can be identified, and the genes encoding them can be cloned they could be inserted into the CCV vector resulting in a multivalent vaccine against both CCV and *E. ictaluri*. Alternatively, genes encoding protective antigens of CCV could be inserted into an attenuated recombinant strain of *E. ictaluri*.

DNA

Vaccination using deoxyribonucleic acid (DNA) is a relatively new technique first described by Felgner and colleagues (Wolff, et al. 1990). The method is basically another approach to the development of subunit vaccines (Fynan, et al. 1993). With this approach, the host is vaccinated with an injection of purified, non-replicating plasmid DNA expressing the pathogen gene under a eucaryotic promoter. The host cells take up the plasmid DNA, express the encoded protein, and mount a subsequent immune response against it. The efficacy of immunization with DNA encoding a protective protein was first described in mice by Liu and colleagues in 1993 (Ulmer, et al. 1993). Both humoral and cell-mediated protective immune responses are inducible by DNA vaccination (Donnelly, et al. 1995).

Direct injection of plasmid DNA has been shown to have vaccine potential in fish. Muscle cells of carp injected with plasmid DNA were shown to have high levels of expression of the encoded protein (Hansen, et al. 1991). Plasmid DNA encoding the luciferase gene was delivered to Rainbow trout muscle cells by particle bombardment (Levine, et al. 1994). Expression of the luciferase gene was detected within 48 hours and continued for 15 days. As little as 0.01 µg of plasmid DNA delivered IM was sufficient for the detection high levels of

luciferase in the muscle of zebra fish and rainbow trout (Hepell and Davis 1996). With a dose of 0.1 µg of DNA, luciferase could be detected in four hours with peak activity in 2.5 days. The results showed gene expression of injected DNA is very efficient in fish, with much higher levels than those observed with mice (Hepell and Davis 1996). Injection of DNA clearly has potential for use in fish vaccination, however, as of yet no one has reported on the immunogenicity of the proteins encoded by the DNA injected in fish.

Vaccine Enhancers

Adjuvants, immunostimulants and vaccine carriers all work to enhance the immune response. Most of these immune enhancers can only be administered by injection, though some have the potential to be used in immersion or oral vaccination (Ellis 1988b).

Adjuvants

Adjuvants are substances that, when combined with antigen, enhance specific and nonspecific immune responses (Kennedy-Stoskopf 1993). Most Oil-based adjuvants and liposomes act as depots or reservoirs for holding the antigens in tissues after injection for slow release (Anderson 1992). An example is Freund's incomplete adjuvant (FIA), which is a saline and mineral oil emulsion that stimulates a local, chronic inflammatory response at the site of injection. If killed *M. tuberculosis* are included in the emulsion the mixture, known as Freund's complete adjuvant (FCA), then T cells will also be stimulated. The muramyl dipeptide (MDP) component of the bacteria stimulates macrophages to produce IL-1, which in turn stimulates the helper T cell responses (Tizard 1992). Freund's complete adjuvant is the most effective and most widely used adjuvant by experimental immunologists. It can only be administered by injection, and it has many undesirable side effects. It has been used successfully in fish though it does induce sterile abscesses by IM injection and granulomata by IP injection. The MDP component of FCA by itself was found to have no adjuvant effect when used in an injectable vaccine in rainbow trout (Ellis 1988b).

Another oil adjuvant, squalene, was tested in an *E. ictaluri* bacterin delivered by IP injection to catfish (Tyler and Klesius 1994a). Though not directly toxic, the squalene was shown

to decrease resistance to *E. ictaluri* infection rather than enhancing resistance. It was suggested that the decreased resistance was due to a depletion of circulating phagocytic cells due to an extensive granulomatous response at the site of the injection (Tyler and Klesius 1994a).

Another type of adjuvant are the metallic salts aluminum potassium sulfate (alum) and aluminum hydroxide. These are also T cell stimulators (Anderson 1992). Alum is used in a colloidal suspension with antigenic material bound to it, and aluminum hydroxide is used with antigen in a gel matrix (Anderson 1992; Kennedy-Stoskopf 1993). Neither alum or aluminum hydroxide enhanced protection in salmon vaccinated by injection, and alum resulted in serious side effects. In contrast, the use of alum in oral and immersion *Vibrio* vaccines in salmon improved antigen uptake and protection levels considerably (Ellis 1988b).

Immunostimulants

Immunostimulators are drugs, chemicals, stressors or actions that elevate the non-specific immune response. They can be used by themselves to activate the nonspecific immune response, or they can be used as an adjuvant in a vaccine to activate the nonspecific immune response and enhance the specific immune response (Anderson 1992). A number of immunostimulants have been tested in fish. Levamisole is an anti-helminth drug used in ruminants. It is also an immunomodulator that stimulates T cells. Levamisole has been used effectively in fish alone as an immunostimulator and in injection and immersion bacterin vaccines as an adjuvant. The proper dosage to use in fish still needs to be determined since the effects of the drug are dose dependent, and high dosages can be immunosuppressive (Anderson 1992).

Some components of bacterial cells are immunostimulatory. The MDP of *M. tuberculosis* is one example, other examples are BCG, FK-565 and LPS. Bacille Calmette Guerin (BCG) is an attenuated strain of *M. bovis* used to vaccinate humans against tuberculosis. It is also a well recognized immunostimulant in mammals. In fish, BCG has not enhanced immune function when used as an adjuvant with a bacterin (Anderson 1992). The immunostimulant FK-565 is a synthetic peptide based on a component from *Streptomyces olivaceogriseus*. It is a T cell stimulator in mammals and has been shown to be an effective immunopotentiator in fish

(Anderson 1992). The LPS from gram negative bacteria is also an immunostimulant. Lipopolysaccharide preparations stimulate non-specific B cell proliferation both *in vitro* and *in vivo* if given in the appropriate doses. Low doses are effective, so bacterin preparations of gram negative bacteria contain their own immunostimulants. Lipopolysaccharide does not appear to have the same endotoxic effect in fish that it does in mammals. Preparations of LPS have been tested in fish and can be useful as immunostimulators or adjuvants (Anderson 1992). Lipopolysaccharide stimulates B cells directly in a T1 manner so it does not generate immunological memory (Arkoosh, et al. 1991).

Animal and plant extracts have been tested as immunostimulators in fish (Anderson 1992). An extract from the marine tunicate *Ecteinascidia turbinata*, called *Ete*, has been used successfully as an immunostimulant and an adjuvant in American eels (*Anguilla rostrata*). An extract from the abalone *Haliotis discus hannai*, called *HDe*, had adjuvant effects in rainbow trout. A fish extract called ISK worked as both an immunostimulant and an adjuvant in rainbow trout when administered by injection or immersion. An extract from the bark of the South American tree *Quilaja saponaria*, called Quil-A, was shown to enhance bacterial clearance of live *Y. ruckeri* when administered to fish by bath. The active ingredients of these extracts are in most cases not well-defined, and their modes of action are not known (Anderson 1992).

Vaccine carriers

Vaccine carriers are vehicles or transporters for a vaccine (Anderson 1992). Bentonite clay (aluminum silicate) and latex beads have been used as inert carriers for fish vaccines. Carriers facilitate engulfment by phagocytes and they may themselves be immunogenic. In comparison studies, antigen bound to latex beads was taken up more readily by fish phagocytes and resulted in higher protection from the vaccine than antigen by itself. The immunogenicity of small molecules (haptens) can be improved by conjugating them to a carrier protein. Keyhole limpet hemocyanin (KLH) is a commonly used TD protein carrier in fish. Dimethylsulfoxide (DMSO) is another carrier tested in fish (Anderson 1992). It was shown to have adjuvant effects when used with an immersion vaccine in rainbow trout (Ellis 1988b).

Liposomes are biodegradable, non-toxic phospholipid vesicles shown to be capable of functioning as delivery vehicles, and as immunological adjuvants (Nakhla, et al. 1997). In mammals, liposome-encapsulated antigens are thought to exhibit enhanced antigen presentation and recruitment of macrophages, which may result in the potentiation of humoral responses and possibly generate T cell mediated responses. Lipopolysaccharide is a TI antigen in mammals and fish, inducing antibody mediated immune responses without the activation of T cells or the development of immunological memory. In a recent study, LPS from *A. salmonicida* was incorporated into liposomes in order to evaluate their potential for enhancing the immunogenicity of LPS in rainbow trout (Nakhla, et al. 1997). The results showed IP injection of liposomal LPS induced sustained humoral immune responses against LPS compared to the humoral response induced against IP injection of free LPS. Since extended duration of an antibody response is a property of immunological memory, the results suggested liposomes were effective in inducing a TD immune response against the LPS TI antigen (Nakhla, et al. 1997).

ESC VACCINES IN CATFISH

Edwardsiella ictaluri is considered a good candidate for vaccine development because it is a homogeneous single strain of bacteria, and catfish that recover from infection are immune to ESC (Plumb 1988; Klesius 1992a). Like other fish pathogens, vaccines against *E. ictaluri* initially focused on using inactivated bacterial preparations without any understanding of factors important in eliciting a protective immune response against ESC. Unfortunately, this approach has not been very successful, and efforts are now being made to identify important antigens. In addition, more recent efforts have recognized the need to stimulate a cell-mediated immune response, though details on the type of cell-mediated immune response (Th vs Tc) that needs to be generated have not been discussed.

The ESC vaccines tested in channel catfish will be reviewed, followed by a review and discussion on strategies for developing new vaccines based on the survival mechanisms of the bacterium and the type of immune response that must be generated by the catfish in order to overcome these mechanisms.

Overview of Tested ESC Vaccines

Inactivated

Vaccines against *E. ictaluri* have primarily focused on the use of killed bacterins. Many vaccination studies have assessed the vaccines efficacy using indirect measurements of the catfish immune response rather than survival following a challenge. In addition, many of the vaccine studies were initiated in order to better understand the catfish immune response to *E. ictaluri*, rather than assessing the effectiveness of a particular vaccine.

The incidence of ESC is highly seasonal, with infections occurring in the spring and fall. The effect of water temperature on the catfish immune response to *E. ictaluri* was investigated in a field study designed to evaluate the feasibility of vaccinating catfish in the fall or early winter (Plumb, et al. 1986). To determine this, the effects of temperature on the longevity and protectiveness of humoral immunity in channel catfish immunized with formalin-killed *E. ictaluri* (ATCC 33202) were examined. Preliminary results indicated channel catfish could be immunized by either injection or immersion during cold temperatures, as long as the fish were held in warm water for 4 to 5 days prior to being moved to cold water (Plumb, et al. 1986).

Consequently, fish were vaccinated with a sonicated or whole cell bacterin administered either by IP injection with adjuvant or a 5 min. immersion and held at 25 °C for four days prior to stocking in outdoor ponds over the winter (Plumb, et al. 1986). Four months later, fish were collected and natural mortalities were assessed. While in the pond, fish vaccinated by immersion with sonicated or whole cells had 27% and 29% mortality respectively, fish vaccinated by injection with sonicated or whole cells had 63% and 68% mortality respectively, and controls had 42% mortality. Mean antibody agglutination titers (Log_2) in these five groups were 2.0, 2.0, 4.0, 5.0 and 0 respectively (Plumb, et al. 1986).

Fish that survived the winter were challenged at 25 °C with virulent *E. ictaluri* (3.9×10^6) by a 6 minute water-borne exposure. Following challenge, fish vaccinated by immersion with sonicated or whole cells had 12% and 25% mortality respectively, fish vaccinated by injection with sonicated or whole cells had 42% and 58% mortality respectively, and controls had 46%

mortality. Mean antibody agglutination titers (Log_2) in the five groups 23 days post-challenge were 6.4, 6.5, 7.0, 6.0 and 5.1 respectively (Plumb, et al. 1986). The relative percent survival ($\text{RPS} = [100 \times (\text{treatment \% mortality} + \text{control \% mortality} - 1) \times 100]$) of the fish vaccinated by immersion with sonicated or whole cells was 74% and 47% respectively, the RPS of the fish vaccinated by injection with sonicated or whole cells was 9% and 0% respectively (Plumb 1988). Results of this study showed the vaccinated fish did develop humoral antibodies and some protection against challenge four months later. Surprisingly, vaccination by injection with an unidentified adjuvant was not protective and vaccination by immersion was protective even though the injected vaccine resulted in higher antibody titers (Plumb, et al. 1986).

In a laboratory study, the efficacy of a formalin-killed *E. ictaluri* whole cell bacterin (ATCC 33202) was investigated using combinations of injection or immersion delivery with and without boosters and FCA (Saeed and Plumb 1986). The animals used in the study were one-year-old pond reared catfish acclimated in the laboratory two weeks prior to experimentation. Vaccine efficacy was measured by serum agglutination antibody titers and immunity to experimental challenge of a lethal dose 80% endpoint (LD_{80}) of virulent *E. ictaluri* administered by IP injection. Multiple injections of whole cells in FCA resulted in high titers (2730) and strong protection (8.3% mortality). Single injections of whole cells in FCA resulted in good titers (1220) and some protection (31.1% mortality). Multiple and single injections of the bacterin in saline resulted in low titers (341 and 158 respectively) and no protection. Multiple and single DI and HI dips in bacterin resulted in low titers (158-248) and no protection (Saeed and Plumb 1986). Multiple and single saline injection controls had titers of 6 and 99 respectively, and percent mortalities of 75% and 66.7% respectively. The fact that the saline controls had positive *E. ictaluri* titers suggests the experimental fish were exposed to *E. ictaluri* prior to being moved from the pond to the laboratory.

The results of this study indicated that both adjuvant and booster immunizations enhanced immunity (Saeed and Plumb 1986). The higher protection seen with the injected vaccine compared to the immersion vaccine is not unexpected, but it does conflict with the

results of the field study described previously (Plumb, et al. 1986). The poor efficacy of the immersion vaccines may have been due to the fact that fish were vaccinated by a water-borne route, but challenged by an unnatural route (IP injection). Vaccination by immersion has been shown to elicit a mucosal antibody response (Lobb 1987). The injection challenge would bypass any mucosal immune responses that may be important in protective immunity against ESC.

In another field study, Vinitnantharat and Plumb (1992) examined factors pertinent to vaccination of catfish with *E. ictaluri*, including kinetics of the immune response, antigen concentration, water temperature and duration of immersion exposure to antigen. The antibody agglutination titers of fish immunized with either cell extract or cell envelope increased gradually 1 week after IP injection and then more rapidly until peaking four weeks after immunization. Antibody titers gradually declined after peaking, but were still detectable 11 weeks after immunization in fish not receiving a booster injection. An anamnestic response immediately followed a booster vaccination administered 6 weeks after the initial vaccination, with an increased in antibody titer for a short period of time (Vinitnantharat and Plumb 1992). Fish in the different vaccination groups were marked by group with fin-clips and stocked in an earthen pond during the experiment. None of the groups had *E. ictaluri* antibodies at the beginning of the study, but fish in the non-vaccinated control group developed antibodies during the experiment, probably due to *E. ictaluri* organisms endemic to the holding pond (Vinitnantharat and Plumb 1992).

The study also showed formalin-killed *E. ictaluri* whole cell bacterin administered by IP injection produced higher antibody titers with 2×10^6 or 2×10^8 cells/fish than with 2×10^4 cells/fish. An IP injection of crude membrane protein produced higher antibody titers with 0.20 or 1.50 mg protein/fish than with 0.02 mg protein/fish (Vinitnantharat and Plumb 1992). In addition, fish held at 25 °C and 30 °C had higher antibody titers than fish held at 20 °C, and longer immersion exposure to antigen resulted in higher antibody titers. Results of the study indicated channel catfish do respond anamnesticly to a booster injection, antigen concentrations can be

optimized, the optimal water temperature for antibody production is 25 °C or above, and immersion exposure to a vaccine should be at least 2 min (Vinitnantharat and Plumb 1992).

The large numbers of fish in production and their low individual value makes injection vaccination of catfish impractical. Consequently, experiments were done to evaluate the effectiveness of an immersion and oral vaccination program with fry and fingerling channel catfish (Thune, et al. 1994). This was done in three large scale field trials. The first trial was comprised of 12 commercial ponds, with three replicates of four treatments at a farm in Mississippi. Catfish were vaccinated with a formalin-killed *E. ictaluri* whole cell bacterin strain 85-58 by immersion vaccination, oral vaccination, or by a combination of immersion vaccination with an oral boost. Results indicated all three treatments resulted in increased survival compared to non-vaccinated controls, but no statistical difference was detected due to the high level of variation between ponds.

A subsequent expanded field trial using the immersion/oral booster protocol was conducted using a formalin-killed *E. ictaluri* whole cell vaccine prepared by Biomed Inc. (Bellview Washington, USA). This study was comprised of 22 ponds at three commercial catfish farms in Louisiana, Mississippi and South Carolina (Thune, et al. 1994). Results of this trial indicated no significant difference between vaccinated and non-vaccinated fish. A change in the way the oral booster was prepared was thought to have effected the results (Thune, et al. 1994). The following year, the field trial was repeated using 26 ponds on five farms. Results of this trial indicated overall mortality in vaccinated fish (41% mortality) was significantly lower than in the non-vaccinated controls (64% mortality). However, the average RPS was only 35%, and the RPS values varied between the five farms with values of 81%, 77%, 27%, 15% and 0% (Thune, et al. 1994). Challenge in these studies was by natural exposure to *E. ictaluri* in the ponds. In addition, fish were exposed to a number of other naturally occurring infectious and parasitic agents, and epizootics by several of these agents did occur in some of the experimental ponds (Thune, et al. 1994).

When vaccine experiments are conducted on catfish held or reared in outdoor ponds, natural exposure to *E. ictaluri* and other infectious agents cannot be controlled. Natural exposure

to *E. ictaluri* results in positive *E. ictaluri* antibody titers in non-vaccinated control fish, and may serve as a natural uncontrolled booster in vaccinated treatment fish. Infections and mortalities due to other infectious agents in control and treatment fish may also interfere with study results. The results of field studies are therefore often equivocal and difficult to compare (Thune, et al. 1997a).

Consequently, a laboratory study was conducted using *E. ictaluri* specific pathogen free (SPF) catfish to compare humoral antibody responses of fish vaccinated by injection, immersion and oral delivery methods (Thune, et al. 1997a). Four different vaccination regimes, including non-vaccinated controls, immersion/oral, late immersion and injection were evaluated for antibody production and protection from challenge in catfish hatched and raised over a 28 week period. The results showed catfish could produce a measurable antibody response against a formalin-killed *E. ictaluri* whole cell bacterin (Biomed) via all of the delivery methods evaluated. Antibody titers, measured by ELISA, were highest in fish vaccinated by injection with a peak titer of 8 (Log_2) at 6-8 weeks post-vaccination (Thune, et al. 1997a). An ELISA titer greater than 8 (Log_2) corresponds to an agglutination titer greater than 256. A previous study has indicated agglutination antibody titers greater than 256 are necessary to ensure protection (Vinitnantharat and Plumb 1993).

Protection from challenge could not be evaluated in four of five challenges in this study due to concurrent infection of *Cytophaga columnaris* (Thune, et al. 1997a). In one challenge that did not have a concurrent *C. columnaris* infection, only the injected group had higher survival than the control with an RPS of 57%. Ninety percent of the vaccinated fish were found to have titers below the 256 reported to be necessary for protection (Vinitnantharat and Plumb 1993), which may have accounted for the low level of protection. Another problem, in addition to having to eliminate four out of five of the challenge studies, was the low percent mortality in the challenged, non-vaccinated controls of the challenge study that was not eliminated. Although the calculated RPS of the injected group was 57, the percent mortality in the non-vaccinated control challenged group was only 33%. Optimal potency testing of fish vaccines requires a

minimum of 60% mortality in non-vaccinated challenged controls (Amend 1981; Ellis 1988a), though some researchers suggest greater than 80% mortality (Ward 1982). This is to assure there are a sufficient number of susceptible fish in the population to allow data analysis and to assume an adequate level of challenge, without overwhelming any protective immunity (Amend 1981). Amend (Amend 1981) suggests a mortality level below 60% in the challenged non-vaccinated control is unsatisfactory and the test should be considered a "no" test and repeated. Although it is not unusual for researchers to report fish vaccine study results with challenge mortalities lower than 60% in the controls, conclusions should not be drawn on the protective capabilities of the vaccine.

Despite logistically difficult and extensive field and laboratory trials by Thune and colleagues (Thune, et al. 1994; Thune, et al. 1997a), the results of vaccination studies in catfish using inactivated *E. ictaluri* bacterins are equivocal. Most of the vaccination studies are marred by uncontrolled exposure of the controls and/or treatment groups to *E. ictaluri* and other infectious or parasitic agents.

Controlled Live

A unique vaccination method was recently used in studies of the catfish protective immune response against ESC (Shoemaker and Klesius 1997; Shoemaker, et al. 1997). With the controlled live vaccination (CLV) method, catfish were exposed to levels of *E. ictaluri* which were apparently high enough to cause infection and induce a protective immune response, but too low to cause disease. The purpose of these studies was to compare immunity against ESC with the specific antibody response and *in vitro* killing of *E. ictaluri* by elicited peritoneal macrophages from catfish immunized by a commercial immersion bacterin (ESC vaccine, Apharma, formerly Biomed Inc.), a commercial oral-fed bacterin (Escogen, Aqua Health Ltd, Charlottetown, PEI, Canada), or controlled live *E. ictaluri* exposure (Shoemaker and Klesius 1997).

Juvenile 17 month old catfish were stocked into laboratory aquaria, with 30 fish per tank and triplicate tanks of each treatment group. The IM and oral vaccination procedures were carried out following the manufacturers' protocols. For CLV, fish were immersed in water

containing 2×10^6 CFU ml⁻¹ live *E. ictaluri* in BHI broth (AL-93-75) for 1 h. After exposure, fish were returned to aquaria. Water flow was stopped for one hour. At 33 days post-vaccination or 18 days post-booster, if administered, fish were challenged with 2×10^7 CFU ml⁻¹ *E. ictaluri* (AL-93-75) at 28 °C for 1 h. Survival after vaccination was 83.3% for CLV, 98.1% for IM and 100% for oral routes of exposure and the controls. Survival after challenge was 100% for CLV, 68.3% for IM, 50% for oral, and 71.7% for the controls. Survival after vaccination was significantly lower in CLV fish, but protection after challenge was significantly higher. Only CLV fish had significantly higher survival than non-vaccinated, challenged controls. Antibody responses and macrophage bactericidal activity were also significantly higher in the CLV group, although antibody responses were assessed using an ELISA assay that only measured the antibody titers against a single *E. ictaluri* exoantigen (Shoemaker and Klesius 1997). Like several of the inactivated vaccine studies discussed previously, percent mortality in non-vaccinated, challenged controls was only 28.3%.

Results of the study showed controlled exposure to *E. ictaluri* provides protection against ESC. It has been suggested that natural exposure of vaccinated catfish to *E. ictaluri* in the pond environment may serve as a natural booster (Thune, et al. 1994). Additionally, this study showed natural exposure to *E. ictaluri* in the pond environment may immunize catfish and result in protection against ESC (Shoemaker and Klesius 1997).

Attenuated

Using genetic engineering, a virulent strain of *E. ictaluri* (93-146) was mutated by creating a deletion, kanamycin resistance gene insertion in the *purA* gene (Lawrence, et al. 1997). The resulting recombinant, adenine auxotrophic *E. ictaluri* strain (LSU-E2) was evaluated in SPF catfish for its virulence, tissue persistence and vaccine efficacy. Attenuation was evaluated by injection, immersion and oral routes of exposure. By the injection route, LSU-E2 had an LD₅₀ of 5.1×10^7 CFU per fish. An LD₅₀ could not be determined for the wild-type strain, but it was <130 CFU per fish, more than five logs₁₀ lower than the LD₅₀ of LSU-E2 (Lawrence, et al. 1997). By the immersion route, mortality was 0% in fish challenged with 1.6×10^7 CFU/ml

of LSU-E2 and 63.6% in fish challenged with 6.5×10^7 CFU/ml of wild-type. By the oral route, mortality was 0% in fish challenged with 1.6×10^8 CFU of LSU-E2 and 20.7% in fish challenged with 1.3×10^8 CFU of wild-type (Lawrence, et al. 1997).

Tissue distribution and persistence of the LSU-E2 and wild-type *E. ictaluri* were evaluated in fish following immersion exposure. Samples of liver, spleen, head kidney and trunk kidney were cultured from individual fish before exposure and 2, 6, 12, 24, 48, 72 and 96 hours post-exposure. Adenine auxotrophic *E. ictaluri* strain LSU-E2 was isolated from at least one internal organ in all to the fish sampled from 2 hours to 48 hours post-exposure, indicating its invasive capabilities were still intact (Lawrence, et al. 1997). Wild-type *E. ictaluri* was isolated from fish sampled up to 96 hours post-exposure, when the experiment was terminated. With both LSU-E2 and wild-type bacteria, the highest numbers were cultured from the head and trunk kidneys and the lowest numbers were cultured from the liver. At all sampling times, the numbers of bacteria isolated were higher for wild-type than for LSU-E2 (Lawrence, et al. 1997).

In vaccine trials, SPF catfish were immunized with a single immersion exposure to 3.7×10^7 CFU/ml of LSU-E2. Feeding activity remained normal and there were no mortalities following vaccination. Twenty-seven days after vaccination, both vaccinated and non-vaccinated fish were challenged by immersion exposure to 5.3×10^4 CFU/ml wild-type *E. ictaluri* (Lawrence, et al. 1997). Resulting mortalities in the two treatment groups were significantly different ($P < 0.01$), with 33.3% average percent mortality in non-vaccinated fish and 11.1% average percent mortality in vaccinated fish. The RPS was 66.3% for vaccinated fish (Lawrence, et al. 1997). The results showed immersion vaccination of catfish with the LSU-E2 strain of *E. ictaluri* provided significant protection against experimental infection with wild-type *E. ictaluri* (Lawrence, et al. 1997).

The attenuated LSU-E2 strain of *E. ictaluri* may have potential as a viable commercial vaccine. However, this study, like that of Thune et al (1997a) had a low percent mortality in challenged, non-vaccinated controls. The vaccination trial should be repeated before firm conclusions about efficacy can be made.

Immersion vaccination is a much more feasible delivery method for the catfish industry than injection vaccination. One of the disadvantages of attenuated vaccines include residual virulence and the potential for reverting back to full virulence. The attenuated strain appears to be stable since no revertants of the LSU-E2 were detected following 30 passes in BHI (Lawrence, et al. 1997). Live vaccines are also more susceptible to contamination by other organisms, and they require more care in preparation, storage and handling to maintain viability (Tizard 1992). If the efficacy of an LSU-E2 immersion vaccine is confirmed, the logistics of preparation, storage and handling of a live vaccine in an aquaculture setting will need to be established before a commercial licence will be granted.

Subunit

With subunit vaccines an antigen of interest is purified from the bacteria or virus. To be effective, the purified antigen must be protective. A protective antigen needs to induce a TD antibody response so memory is generated. A vaccine consisting of purified *E. ictaluri* LPS is the only ESC subunit vaccine to be tested for both immune response and efficacy in catfish (Saeed 1983).

In a laboratory study, efficacy of a phenol extracted *E. ictaluri* (ATCC 33202) LPS subunit vaccine was investigated using combinations of IP injection with and without boosters and FCA (Saeed and Plumb 1986). Animals used in the study were one-year-old pond reared catfish acclimated in the laboratory two weeks prior to experimentation. Vaccine efficacy was measured by serum agglutination antibody titers and immunity to experimental challenge of a lethal dose 80% endpoint (LD_{80}) of virulent *E. ictaluri* administered by IP injection. Multiple injections of LPS in adjuvant and a single injection of LPS in adjuvant both resulted in high titers (1391 and 2048 respectively) and strong protection (3.3% and 20% mortality respectively). Results of multiple injections of LPS in FCA were not significantly different from those of a single injection of LPS in FCA, but both were significantly higher than titers (136 and 77 respectively) and protection (36.7 and 80% mortality) seen with multiple and single injections of LPS in saline. Protection of all but the single injection of LPS in saline (80% mortality) was significantly higher than

multiple and single injection saline controls (70 and 80% mortality respectively). Titers elicited by single and multiple injections of saline (4 and 5 respectively) were significantly lower than any other treatments (Saeed and Plumb 1986). The fact that saline controls had positive *E. ictaluri* titers suggests the experimental fish were exposed to *E. ictaluri* prior to being moved from the pond to the laboratory.

Results of this study indicated both adjuvant and booster immunizations enhanced immunity, although the effects of enhancement were much greater with FCA than booster immunizations (Saeed and Plumb 1986). The protection level of multiple and single injections of LPS in FCA (3.3% and 20% mortality respectively) was similar to that of multiple and single injections of formalin-killed *E. ictaluri* whole cell bacterin in FCA (8.3% and 31.3% mortality respectively). Multiple injections of LPS in saline had higher protection (36.7% mortality) than multiple injections of bacterin in saline (76.7%; Saeed and Plumb 1986).

It should not be surprising that injections of LPS with FCA were more protective than injections of LPS in saline. Lipopolysaccharide stimulates B cells directly in a TI manner so it does not generate immunological memory (Arkoosh, et al. 1991). The fact that multiple injections of LPS in saline had some level of protection was probably due to previous exposure of the experimental fish to *E. ictaluri*. Addition of FCA with LPS results in a TD response. This is because the MDP component of the bacteria in FCA stimulates macrophages to produce IL-1, which in turn stimulates helper T cell responses (Tizard 1992). The fact that LPS was more protective than bacterin when both were administered in multiple injections with adjuvant suggests LPS is a protective antigen, but only if a TD response can be generated. This requires the use of a T cell stimulating adjuvant such as FCA, conjugation to a TD carrier such as KLH, encapsulation in liposomal vesicles, or use of a live attenuated strain.

In another study, several immunogenic *E. ictaluri* antigens were identified in SDS-PAGE Western blots probed with sera from *E. ictaluri* infected catfish and *E. ictaluri* immunized, protected catfish (Plumb and Klesius 1988). In strips of an SDS-PAGE Western blot of *E. ictaluri* (ATCC 33202) lysate probed with sera from seven individual *E. ictaluri* catfish, the most strongly

recognized band had an approximate molecular weight of 34 KD. The 34 KD band was recognized strongly by all seven fish indicating it may be an immunodominant antigen. In SDS-PAGE Western blots of 14 strains of *E. ictaluri* probed with pooled sera from *E. ictaluri* infected and *E. ictaluri* immunized, protected fish, the 34 KD band was strongly recognized as well as a 60 KD band in all 14 strains (Plumb and Klesius 1988). A monoclonal antibody (mAb AA224) was used to show that both the 34 KD antigen (reported as 36 KD in this study) and 60 KD antigen were located on the *E. ictaluri* cell surface (Klesius and Horst 1991).

In a subsequent study, proteins from the outer membrane of *E. ictaluri* strain ALG-88-122 were isolated (Vinitnantharat, et al. 1993). The outer membrane of *E. ictaluri* on SDS-PAGE contained three major bands with apparent MWs of 18.4, 36, and 42.5 KD. Only the 36 KD protein persisted through repeated passages of the bacterium on BHI agar. The 36 KD protein was purified and its immunogenicity was evaluated and compared to cell extracts and crude membrane preparations of *E. ictaluri* (Vinitnantharat, et al. 1993). Forty juvenile catfish were acclimated and held in a single laboratory tank through-out the experiment. Fish were divided into four groups, using a fin-clip method of identification. One group of 10 fish served as a control, with each fish receiving 0.1 ml of Tris-EDTA by IP injection. Of the remaining fish, 10 received 0.1 mg of cell extract, 10 received 0.1 mg of crude membrane and 10 received .02 mg of 36 KD protein in Tris-EDTA by IP injection. Each fish was bled weekly for five weeks and anti-*E. ictaluri* titers were measured by microagglutination (Vinitnantharat, et al. 1993). Results showed all of the antigen preparations were strongly immunogenic, with peak antibody titers being reached 3 weeks after injection. The antibody response to each antigen preparation was significantly different from the others ($P > .05$), with the highest titer being elicited by the crude membrane preparation (3.2 approximate Log_{10} titer), followed by the cell lysate (2.8 approximate Log_{10} titer), than the 36 KD protein (2.4 approximate Log_{10} titer; Vinitnantharat, et al. 1993). Although titer was significantly lower with the 36 KD protein, it was administered at one-fifth the dosage of the other two antigen preparations. At a fraction of the dosage, the 36 KD protein was highly immunogenic and able to induce a respectable antibody response compared to that of the

other two antigen preparations. The protective capabilities of the three antigen preparations were not evaluated (Vinitnantharat, et al. 1993).

Cross-Reactive Protective Antigens

All bacterial species possess unique antigens, however a surprisingly high number of antigens are shared among various bacterial species (Kaufmann 1993). Catfish *E. ictaluri* antibodies have been shown to have a high amount of cross-reactivity with a rough mutant of *E. coli* 0111:B4 (Tyler and Klesius 1994c). Since a portion of the cross-reactivity could be absorbed out by LPS from a rough mutant of *S. typhimurium* TV119, it was concluded that natural exposure to *E. ictaluri* induces both specific and cross-reactive antibodies, and much of the cross-reactivity is to the homologous core region of the LPS layer (Tyler and Klesius 1994c).

In order to determine if cross-reactive antigens of *E. coli* were protective, yearling catfish from *E. ictaluri*-free stock were immunized with formalin-killed whole cells of a rough mutant strain of *E. coli* strain (J5; Tyler and Klesius 1994b). Four, non-replicated groups of 18-25 fish were bled than vaccinated. Group one fish were vaccinated with aluminum hydroxide-precipitated bacterin in phosphate buffered saline (PBS) by IP injection, group 2 fish were vaccinated with bacterin in PBS by IP injection, group 3 fish were vaccinated with aluminum hydroxide-precipitated bacterin in PBS by IM injection, and group 4 fish received PBS by IP injection. On day 20 post-vaccination, fish were bled and then challenged with virulent *E. ictaluri*. Survival was 92% in fish that received *E. coli* with adjuvant IP, 77% in fish that received *E. coli* with adjuvant IM, 54% in fish that receive *E. coli* only IP, and 56% in fish that received PBS IP. Survival was significantly higher ($P<.05$) in fish that received *E. coli* in alum IP compared to control fish that received PBS IP. There were no significant differences between the other treatments (Tyler and Klesius 1994b). There was no clear relationship between ELISA titers and survival. The three vaccinated groups of surviving fish had increased titers to both *E. ictaluri* and *E. coli* 20 days-post vaccination, with fish that received *E. coli* IP only having the highest titers. However, all four groups had pre-vaccination ELISA titers to both *E. ictaluri* and *E. coli*, and control fish also had an increase in titer 20 days post-vaccination (Tyler and Klesius 1994b). This

suggests that the experimental fish had been previously exposed to *E. ictaluri*, and there may have been exposure during the experiment since control titers increased. Although the results were marred by indications of previous *E. ictaluri* exposure, they suggested the R-mutant vaccine with adjuvant provided a level of protection against *E. ictaluri* similar to that reported for *E. ictaluri* bacterins (Tyler and Klesius 1994b).

An R-mutant was used because it was assumed LPS core antigens were important in cross-reactive immunity. Inclusion of a treatment group of fish vaccinated with a smooth strain of *E. coli* may have helped show if this assumption was justified since absorption studies have shown antibodies to the core LPS antigen are not the only anti-*E. ictaluri* antibodies cross-reactive with *E. coli* surface antigens (Tyler and Klesius 1994c). In mammals, lipid A from many bacterial species has been shown to have a conserved immunodominant epitope. Thus, the lipid A segment of LPS is considered an ideal target for the generation of antibodies that might cross-react with LPS from different bacterial species (Nakhla, et al. 1997).

Strategies for Developing New ESC Vaccines

Survival Mechanisms of *E. ictaluri*

It is now recognized that a better understanding of catfish protective immune response is needed in order to develop a successful vaccine. In order to understand the protective immune response, any virulence factors or survival mechanisms that the pathogen has should also be understood.

Virulence factors. No specific virulence factors have been identified for *E. ictaluri*. The bacterium is negative for the production of numerous enzymes, though it does produce chondroitinase, an enzyme that degrades the chondroitin sulfate component of cartilage, it has a cell-bound hemolysin, and it has a mannose-resistant and mannose-sensitive hemagglutinin (Waltman, et al. 1986; Wong, et al. 1989). Virulent strains of *E. ictaluri* were shown to have quantitative differences in extracellular products compared to avirulent strains. Virulent isolates had greater amounts of polysaccharide material and surface proteins, and higher chondroitinase activity than avirulent strains (Stanley, et al. 1994).

Extracellular and/or intracellular lifestyle. Although ESC is generally an acute septicemia that develops quickly, there is also observational evidence *E. ictaluri* is a facultative intracellular bacteria (Miyazaki and Plumb 1985; Baldwin and Newton 1993). Facultative intracellular bacterial parasites are able to multiply outside of phagocytes and, under certain conditions, within phagocytes following engulfment (Steele, et al. 1977). Invasion of host cells is not restricted to intracellular bacteria, however, and transient passage through epithelial cells is a common invasion mechanism of both intracellular and extracellular bacteria (Kaufmann 1993).

Based on observations on the type of infection it causes and the host responses it elicits in catfish, the type of intracellular lifestyle *E. ictaluri* leads can be postulated and compared with those of other facultative intracellular bacteria. Protective immunity is much better understood in facultative intracellular bacterial species important in human and veterinary medicine, and these bacteria may serve as models for determining which immune responses might be important in protection against ESC. The hallmarks of an "ideal" intracellular bacterium include a primarily intracellular lifestyle, T cell dependence and antibody independence, low or no toxicity, a chronic course of disease, granulomatous tissue reactions, and DTH . These hallmarks, with respect to *E. ictaluri* are discussed below.

The first hallmark of intracellular bacteria is their ability to survive and sometimes multiply within phagocytes. Numerous pathological studies of *E. ictaluri* have described the presence of macrophages or neutrophils containing bacteria and in some cases undergoing degeneration (Blazer, et al. 1985; Miyazaki and Plumb 1985; Shotts, et al. 1986; Newton, et al. 1989; Baldwin and Newton 1993; Morrison and Plumb 1994). Several investigators have reported dividing *E. ictaluri* cells within phagocytic vacuoles of catfish macrophages and neutrophils. These reports were based on observations in histological sections of neutrophils containing *E. ictaluri* cells in apparent binary fission (Miyazaki and Plumb 1985), and leukocytes containing bacteria that were apposed end to end and incompletely separated (Baldwin and Newton 1993). Experimental evidence is still needed to verify *E. ictaluri* is actually replicating

within the phagocytes. There is no evidence to suggest *E. ictaluri* escapes from the phagosome to enter the macrophage cytoplasm. *Edwardsiella ictaluri* has also been observed as free cells within catfish tissues (Miyazaki and Plumb 1985; Morrison and Plumb 1994). In catfish infected by intragastric intubation, intracellular bacteria were not observed during the acute phase of infection (days 1-7 post-exposure; Shotts, et al. 1986). However, in a different intragastric intubation exposure study, bacteria were observed in macrophages early in the infection (Baldwin and Newton 1993).

The second hallmark of intracellular bacteria is a T cell dependent, antibody independent host immune response. The T cells mediate protection against intracellular bacteria by interacting with infected host cells. In contrast, antibodies interact directly with the bacteria. Antibodies have little effect on intracellular bacteria once they are protected inside the macrophage (Kaufmann 1993). The role of T cells in the immune response of catfish against *E. ictaluri* has not been investigated, but antibody and phagocyte responses have been studied and were discussed previously. The role of specific antibodies in protection has not been determined definitively, but they do appear to play a role in the immune response to *E. ictaluri*, particularly in high titer (Vinitnantharat and Plumb 1993) or when combined with phagocytic cells (Scott, et al. 1985; Sheldon and Blazer 1991; Waterstrat, et al. 1991; Wise, et al. 1993; Shoemaker, et al. 1997).

The third hallmark of intracellular bacteria is low or no toxicity to the host. Pathology to the host by intracellular parasites is primarily due to the immune response itself. Pathology to the host by extracellular bacteria is primarily due to toxins and enzymes which cause tissue damage directly. *Edwardsiella ictaluri* has low toxicity to the host catfish. It has tested negative for production of a number of extracellular enzymes, but it does have an enzyme that degrades chondroitin sulfate, a major component of cartilage (Waltman, et al. 1986). This enzyme is thought to play a role in the HH lesions that develop in chronic infections (Shotts, et al. 1986).

The fourth hallmark of intracellular bacteria is a chronic course of disease. This occurs because the bacteria coexist in their cellular environment for long periods of time, resulting in

a longer incubation time and a chronic disease course (Kaufmann 1993). Extracellular bacteria typically cause acute diseases that develop soon after entry into the host, and they are terminated once the immune response has developed (Kaufmann 1993). *Edwardsiella ictaluri* infections can be manifested in both acute and chronic forms. Epizootics of ESC are generally acute septicemias that develop very quickly (Thune, et al. 1997b), with chronic ESC developing 2–4 weeks after infection (Shotts, et al. 1986).

The fifth hallmark of intracellular bacteria is a granulomatous tissue reaction in the host. Both protection and pathology caused by intracellular bacteria centers around the granulomatous lesions. In contrast, the tissue reactions against extracellular bacteria are purulent and lead to abscesses or systemic reactions (Kaufmann 1993). Granulomatous inflammation is seen in a number of host tissues with both the acute and chronic forms of ESC. Fish differ from mammals in that they produce hemorrhagic liquefaction rather than pus or purulent abscesses (Ellis 1981). Both petechial hemorrhaging and systemic bacteremia are seen in the acute form of ESC.

The sixth hallmark of intracellular bacteria is that infection is accompanied by a DTH response in the host. Fish can exhibit typical DTH skin reactions against *Mycobacterium* and other bacterial antigens (Manning and Nakanishi 1996). The DTH skin response of catfish following infection with *E. ictaluri* does not appear to have been investigated.

The six hallmarks listed above are for the "ideal" intracellular bacteria. The ideal intracellular bacteria does not actually exist, but the organism that comes closest is *M. tuberculosis* (Kaufmann 1993). Many intracellular bacteria do not have one or more of the hallmarks. For example, *Listeria monocytogenes* infections in experimental mice are acute, and antibodies participate in the protective immune response against *Salmonella typhi*, *S. paratyphi* and *Brucella abortus* (Tizard 1992; Kaufmann 1993).

With *E. ictaluri*, the evidence seems to suggest that during an acute infection it may occur both extracellularly and intracellularly. Resistance to complement mediated lysis by the alternate pathway may be one of the mechanisms used by *E. ictaluri* that allows it to survive extracellularly in a naive host (Ourth and Bachinski 1987). The apparent ability *E. ictaluri* to

survive within phagocytes may allow it to disseminate through-out the host more quickly, and may allow it to evade the non-specific, innate immune responses of the catfish upon initial infection.

Relevant Catfish Immune Responses

Factors likely to be important in a protective immune response against *E. ictaluri* can be postulated based on the protective immune responses against human and veterinary intracellular bacteria that have survival mechanisms similar to those of *E. ictaluri*.

Humoral. Antibodies are important in the protective immune responses against some intracellular bacteria. In typhoid, caused by *S. typhi* and *S. paratyphi*, antibodies are thought to participate in the protective immune response (Kaufmann 1993). Antibodies also contribute to resistance to *Brucella abortus*, the cause of contagious abortion in cattle (Tizard 1992). If opsonized by antibodies, phagocytized *B. abortus* stimulates a phagocyte respiratory burst and is destroyed by cattle neutrophils. If it is not opsonized, *B. abortus* fails to trigger this response and is able to survive within neutrophils (Tizard 1992). *Edwardsiella ictaluri* appears to be similar to *B. abortus* in that opsonization with immune serum significantly increases bactericidal activity of the catfish macrophage (Scott, et al. 1985; Sheldon and Blazer 1991; Waterstrat, et al. 1991; Wise, et al. 1993; Shoemaker, et al. 1997).

Cell mediated phagocytic responses. Protection against intracellular bacteria is cell mediated. Although monocytes and resting macrophages from naive animals are effective phagocytes, they are unable to kill intracellular bacteria. Killing ability is acquired about 10 days after the onset of infection after macrophages have gone through several levels of activation. When monocytes first move into inflamed tissues they develop increased levels of lysosomal enzymes, increased phagocytic activity, and increased expression of antibody, complement and transferrin receptors. These cells are known as inflammatory macrophages (Tizard 1992). Inflammatory macrophages can be further stimulated to become activated macrophages. Macrophage activation is mediated by cytokines such as INF- γ and by specific antibodies on opsonized bacteria that bind to FcR receptors on the macrophage (Tizard 1992). Activation by INF- γ results

in the transition of macrophages from cells that support microbial replication into effector cells that terminate or at least restrict microbial survival (Kaufmann 1993). Immunity due to activated macrophages is usually short-lived and non-specific since cells are able to kill a wide range of normally resistant bacteria. However, activated macrophages also express more MHC class II antigen on their surface and thus have an enhanced ability to process antigen and present it to CD4 T cells for the generation of an immunological memory response (Tizard 1992). Since opsonization of *E. ictaluri* cells with specific antibodies significantly increases the bactericidal activity of catfish macrophages, it appears that specific antibody production and activation of macrophages via binding of FcR receptors may be important in the catfish immune response to ESC.

The T cells are the central mediators of protection against intracellular bacteria. Both MHC class I-restricted CD8 T cells and MHC class II-restricted CD4 T cells may participate in acquired resistance against intracellular bacteria, depending on where they reside within the macrophage (Kaufmann 1993). If an intracellular bacteria is able to escape the macrophage phagosome and enter the cytoplasm, its antigens are entered into the MHC class I processing pathway which results in activation of cytotoxic CD8 T cells. If an intracellular bacteria remains in the macrophage phagosome, its antigens are preferentially entered into the MHC class II processing pathway which results in activation of helper CD4 T cells. Additional mechanisms are thought to exist since CD8 cells against organisms such as *M. bovis* and *S. typhimurium*, which appear to remain in the phagosome, have been isolated (Kaufmann 1993). Since there have been no observations of intracellular *E. ictaluri* cells outside of phagosomal vesicles, the MHC class II antigen processing pathway and subsequent activation of helper T cells is probably the important T cell element of the catfish immune response against ESC. There is no evidence to suggest that cytotoxic T cells are involved, although the catfish T cell response to *E. ictaluri* has not been investigated. A protective helper T cell memory response against *E. ictaluri* might be expected to involve a faster stronger activation of T cells upon recognition of *E. ictaluri* antigen

presented in the context of MHC class II antigen, resulting in IFN- γ production and activation and bactericidal killing of macrophages.

Possible Approaches for Future ESC Vaccines

The immune responses that may be postulated to be the most important in resulting in protection against *E. ictaluri* include specific antibodies, activated macrophages and helper T cells. A vaccine designed to stimulate all of these responses should theoretically result in protective immunity against *E. ictaluri*. This requires a vaccine that can stimulate both humoral and cell mediated immune responses. An attenuated strain of *E. ictaluri* is the most obvious choice of vaccination method since it does not require identification of the protective B and T cell epitopes. Another alternative may be a subunit vaccine of a protective antigen such as LPS or some as yet unidentified protein in liposomal vesicles. Cross-reactive vaccination with another gram negative bacteria may be another alternative, since cross-reactive antibodies such as bacterial heat shock proteins are often conserved T cell antigens (Kaufmann 1993). Using the CCV vector to deliver *E. ictaluri* genes encoding protective antigens could result in protection against both CCV and *E. ictaluri*, although virally encoded antigens would be expressed in the context MHC class I antigens rather than MHC class II. The limitations of any of these approaches will be whether an efficacious vaccine can be economically produced, stored, handled and administered in an aquaculture setting.

Hypothesis and Objectives

The hypothesis of this research was that catfish immunity to ESC is due to humoral or cellular immune responses elicited by protective *E. ictaluri* antigens, and the identification of one or more of these protective antigens could lead to the development of an effective ESC vaccine.

The overall goal was to generate and characterize a pool of candidate antigens and determine if they were protective against ESC. Individual objectives of the research were to: (1) Expand and clarify the identity of antigenic proteins of *E. ictaluri*, (2) generate a pool of cloned antigenic proteins of *E. ictaluri*, (3) characterize the encoded genes and expressed cloned

antigenic *E. ictaluri* proteins, and (4) Evaluate the protective capabilities of the cloned antigenic *E. ictaluri* proteins.

CHAPTER 2: EVALUATION OF THE EXPRESSION OF ANTIGENIC AND NON-ANTIGENIC PROTEINS OF *EDWARDSIELLA ICTALURI* UNDER DIFFERENT CONDITIONS OF GROWTH USING TWO-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS

Whole cell lysates (WCLs) of *Edwardsiella ictaluri* cells grown in brain-heart infusion broth (BHI) and defined minimal media broth (MM19) were analyzed using denatured (D) and native (N) two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). In D2D-PAGE, there was no difference in expression of antigenic and non-antigenic proteins between two virulent strains or between growth temperatures of 25 and 30 °C for cells grown in BHI. Cells grown in MM19 media, however, expressed a unique 57 KD protein not seen in BHI WCLs. Pooled convalescent catfish serum (CCS) recognized 39 bands and 90 spots in western blots of BHI and MM19 WCLs. The same antigenic bands and spots recognized in BHI grown WCLs were detected in the MM19 grown WCL, with the exception of the unique 57 KD protein which was only recognized in the MM19 WCL. Additionally, two other antigenic spots of 58 and 71 KD had differences in level of expression between the MM19 and BHI WCLs. Comparisons of western blots with total protein stained gels showed each of the antigenic bands were resolved into multiple spots that included both antigenic and non-antigenic proteins. In N2D-PAGE, there was no difference in expression of antigenic and non-antigenic proteins between strains or at different growth temperatures for cells grown in BHI. The MM19-grown WCL had differences in protein separation compared to BHI-grown WCLs. Five antigenic spots were recognized by CCS in N2D-PAGE western blots of BHI-grown WCLs. The same five spots were identified in MM19-grown WCLs along with seven additional spots unique to the MM19-grown WCL. These results show antigenic proteins bands of *E. ictaluri* separated by one-dimensional SDS-PAGE may actually consist of one or more antigenic and non-antigenic proteins. In addition, strain and culture temperature does not effect expression of antigenic proteins, but culture media does. Recognition of the unique 57 KD protein by CCS demonstrates it is expressed during an *E. ictaluri* infection. Since this protein was not expressed in BHI, the MM19 media may be more analogous to the *in vivo* environment.

INTRODUCTION

Edwardsiella ictaluri, causative agent of enteric septicemia of catfish (ESC), is the primary bacterial pathogen of commercially produced channel catfish (*Ictalurus punctatus*) in the Southeastern United States (Thune 1991). Although several studies have evaluated the utility of vaccination to prevent ESC, protection from disease has been inconsistent (Plumb, et al. 1986; Saeed and Plumb 1986; Thune, et al. 1994; Thune, et al. 1997a), possibly because of our lack of knowledge concerning antigens important in eliciting a protective immune response. Generation of a protective immune response is contingent on proper presentation of appropriate antigens. To develop a successful ESC vaccine, it may be necessary to identify protective antigens. Although this can be a difficult process, the first step in identifying protective antigens is to identify candidate bacterial proteins antigenic in the host, that is, proteins that react with host antibodies following recovery from infection. Although an antibody response does not necessarily correspond with protective immunity, identification of proteins that are antigenic may narrow the field of possible protective antigens for further studies.

Immunodominant and strongly recognized antigens of *E. ictaluri* have been identified previously from *E. ictaluri* whole cell lysates (WCLs) using one-dimensional sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and colorimetric western blot analysis with catfish immune sera. Plumb and Klesius (1988) identified immunodominant bands with estimated molecular weights of 34 and 60 KD in the reference strain ATCC 33202 using pooled sera from infected catfish and from immunized, protected catfish. In another study, pooled convalescent catfish serum strongly recognized a 39 KD band in virulent strain AL83189 (Baldwin, et al. 1997). Variation in the results of these studies may have been due to differences in the bacterial strain used, culture temperatures, serum source, or differences in sample preparation.

Other researchers have used denatured two-dimensional (2D)-PAGE to evaluate differences in protein induction under various stress conditions, showing multiple proteins are repressed or induced under different conditions of growth (Kwaik, et al. 1993; Rafie-Kolpin, et al.

1996). Protein expression under various culture conditions has not been evaluated in *E. ictaluri*. Previous studies evaluating antigen expression of *E. ictaluri* used enriched culture media such as blood agar, BHI broth, or trypticase soy agar, and different researchers have used different culture temperatures and different bacterial strains (Plumb and Klesius 1988; Baldwin 1997). Antigenic protein expression of *E. ictaluri* has not been evaluated in the defined minimal media (MM19) described by Collins and Thune (1996), or compared between other culture conditions such as differences in temperature.

The goals of this research were two-fold. The first goal was to determine if antigenic *E. ictaluri* protein bands identified using SDS-PAGE and western blot analysis consisted of single proteins or multiple proteins with the same molecular weight. This was accomplished using native and denatured 2D-PAGE (O'Farrell 1975) and enhanced chemiluminescent (ECL) western blot analysis. The second goal of the research was to determine if differences in expression of antigenic proteins could be due to differences in strains or culture conditions. To determine this, antigens of two virulent strains, effect of growth temperature on antigenic protein expression, and antigenic protein expression in enriched versus minimal media were compared using 2D-PAGE and ECL western blotting.

MATERIALS AND METHODS

Bacterial Strains and Media

Virulent *E. ictaluri* strains 92-266 and 93-146 were obtained from the Aquatic Animal Diagnostic Laboratory at the School of Veterinary Medicine, Louisiana State University, Baton Rouge Louisiana, USA. These strains were originally isolated from moribund catfish of natural ESC outbreaks in Louisiana.

Whole Cell Lysate (WCL) Preparation

Bacteria were harvested by centrifugation at 3800 x gravity for 15 min at 4 °C and washed three times in phosphate buffered saline (PBS), pH 7.3. Cells were resuspended in sterile distilled deionized (DDI) water to a standardized volume of 0.1 mL of cell pellet per mL and sonicated at 40 W at 50% duty cycle for approximately 8 min, or until the cell suspensions

changed from milky to clear. After 1 hr at 4 °C, sonicated cells were centrifuged at 12,000 x gravity for 30 min at 4 °C, and 10% sodium ethylmercurithiosalicylate (thimerosal; Sigma Chemical Co., St. Louis Missouri, USA) was added to the supernatant to achieve a final concentration of 0.01% thimerosal. The supernatant was then divided into aliquots and stored at -80 °C.

Growth curves, with growth measured in Klett units, were determined for different growth temperatures and media used in this study. Preliminary results indicated no differences in protein band patterns in SDS-PAGE gels comparing WCLs prepared from 92-266 cells grown at 30 °C or 25 °C and harvested at either early-log, middle-log, late-log or stationary phases of growth (data not shown). Because there was no difference in proteins at different stages of growth, and cell densities were highest at late-log and stationary phases, WCLs used in this study were made from cells harvested at these points.

Lysates were prepared from 92-266 cells grown at both 25 °C and 30 °C, and 93-146 cells grown at 25 °C in brain-heart infusion broth (BHI; Difco Laboratories, Detroit Michigan, USA) to the late-log phase of growth (about 10^8 colony forming units (CFU) per mL). Strain 93-146 was also grown at 25 °C to the stationary (about 10^7 CFU per mL) in a defined minimal media broth (MM19; Collins and Thune 1996). A protein assay (Bio-Rad Laboratories, Richmond California, USA) was used to confirm protein concentrations of the four WCLs were comparable (6.8 ± 0.82 mg/mL). Preliminary results indicated high molecular weight proteins were lost if the WCLs were stored at 4 °C. Consequently, all WCL samples were stored at -80 °C until immediately prior to use. On the day of analysis, samples were thawed and diluted 1:10 in DDI water.

Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE)

The 2D-PAGE was performed using Bio-Rad mini-gel apparatus, following the manufacturer's protocols. First dimension isoelectric focusing (IEF) was performed in non-heparinized micro-hematocrit tubes. In denatured 2D-PAGE (D2D-PAGE), IEF tube gels were 6 cm long and consisted of 4% acrylamide with 9.2 M urea, 2.0% Triton® X-100, 1.6% pH 5/7 ampholyte (Bio-Rad) and 0.4% pH 3/10 ampholyte (Bio-Rad). Diluted WCLs were mixed with an equal volume of IEF sample buffer containing 9.5 M urea, 2.0% Triton® X-100, 5% 2-mercaptoethanol (2ME),

1.6% pH 5/7 ampholyte and 0.4% pH 3/10 ampholyte. Tube gels were loaded with 25 μ L of WCL in IEF sample buffer, 5 μ L D2D-PAGE standards (Bio-Rad), or both, then overlaid with 25 μ L of overlay buffer (9 M urea, 0.8% pH 5/7 ampholyte, 0.2% pH 3/10 ampholyte, 0.25% bromophenol blue). In native 2D-PAGE (N2D-PAGE), first dimension IEF had the same ampholyte and acrylamide concentrations as in denatured IEF, but tube gels lacked urea and Triton® X-100, IEF sample buffer lacked urea, Triton® X-100 and 2ME, and overlay buffer lacked urea. Standards were not run in native IEF. First dimension IEF was run for 10 min at 500 V constant followed by 3.5 h at 750 V constant.

Second dimension 2D-PAGE was performed in 1.0 mm thick, 7 cm x 8 cm slab gels. In D2D-PAGE, second dimension gels consisted of a separating gel (12% acrylamide, .0375 M Tris-HCl, pH 8.8, 0.1% SDS) and a stacking gel (4% acrylamide, 0.125 M Tris-HCl, pH 6.8, 0.1% SDS). Tube gels were extruded from the micro-hematocrit tubes, laid in the trough of the stacking gel, and overlaid with 100 μ L SDS reducing buffer (.0625 M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2ME, 0.13 % bromophenol blue). The reference lane was loaded with 10 μ L 1:10 WCL sample or 1:10 low molecular weight (LMW) silver stain standard (Bio-Rad) diluted 1:4 in SDS reducing buffer and heated to 95°C for 5 min. Denatured PAGE gels were run at 200 V constant for approximately 42 min. Electrophoresis was stopped when the tracking dye was approximately 5 mm from the bottom of the gel. Second dimension N2D-PAGE differed from D2D-PAGE in that the separating and stacking gels consisted of 8% acrylamide and lacked SDS, the native sample buffer did not contain SDS or 2ME, samples were not heated, and no standards were run. Native PAGE gels were run at 12 W constant for approximately 33 min. Following second dimension PAGE, proteins were either silver stained or western blotted.

Total Protein Staining

Total proteins in gels were stained with a rapid silver stain (ICN Radiochemicals, Irvine California, USA) following the ICN protocol, except gels were soaked in 3.5% sulfosalicylic acid, 11.5% trichloroacetic acid solution for one hour and rinsed in DDI water to remove ampholytes before the standard staining procedures. Following the final development step, gels were soaked in 0.1% acetic acid for 10 min and washed in DDI water to decrease background coloration.

Molecular Weight Estimates of Spots and Bands

Band molecular weights were determined from SDS-PAGE gels loaded with BHI and MM19 grown WCLs and LMW standards in the sample lanes. Preliminary 2D-PAGE gels run with LMW standards in the sample lane and 2D-PAGE standards in the IEF tube gels showed that bands and spots of like molecular weights lined up with each other in the gel. The 2D-PAGE standards also showed that the pH gradient of IEF tube gels ranged from pH 4.5 to 6.6. Molecular weights of spots were determined from D2D-PAGE gels of WCLs with LMW standards in the sample lane. Molecular weights were approximated following the methods of Bollag and Edelstein (1991). Relative mobility factors (Rf) were calculated for each molecular weight standard. Molecular weight of each unknown was determined by fitting the unknown Rf into a linear regression equation established using the known \log_{10} molecular weight and Rf values of the standards. The pI values reported for antigenic proteins are subjective approximations reported for comparative purposes only.

Identification of Antigenic Proteins by Western Blot Analysis

Proteins were electrophoretically transferred from unstained gels to 0.45 μ m nitrocellulose membranes at 100 V constant for 1 h in 25 mM Tris, 192 mM glycine and 20% v/v methanol, pH 8.3. Nitrocellulose blots were air-dried and stored in the dark until immunodetection. Proteins blotted to nitrocellulose were probed with pooled convalescent catfish serum (CCS) collected from catfish recovered from natural *E. ictaluri* infections that had occurred at five catfish farms in Louisiana. The five strains isolated from these outbreaks included 92-266. Pooled CCS was made up of sera from 15 fish (3 from each farm) with high ELISA and agglutination titers of *E. ictaluri* antibodies. Normal catfish serum (NCS) was collected and pooled from catfish hatched and reared in the Specific-Pathogen-Free Laboratory at the School of Veterinary Medicine, LSU. The NCS, from fish with no previous exposure to *E. ictaluri*, was used as a primary antibody control. A portion of CCS was absorbed with lipopolysaccharide (LPS) to remove anti-LPS antibodies, this LPS absorbed sera was designated CCS-LA. Absorption was accomplished by mixing CCS with an equal volume of 1 mg/ml purified *E. ictaluri*

LPS, strain AL83189, (Baldwin, et al. 1997), suspended in DDI water, and incubating at room temperature for 2 h on a rocker. Prior to use, all catfish sera were heat inactivated at 56 °C for 30 min and were diluted to a final concentration of 1:50 to 1:200.

Monoclonal mouse anti-catfish IgM (Miller, et al. 1987) was used as the secondary antibody. The mAb 9E1 was a component of harvested hybridoma 9E1 cell culture supernatant, which was diluted 1:4 and used directly on nitrocellulose membranes. Tertiary antibody for initial blots was protein A-affinity-purified goat anti-mouse IgG horseradish peroxidase conjugate (GAM-HRP; Bio-Rad) at 1:3000, while antibody for subsequent blots was affinity isolated, antigen-specific goat anti-mouse IgG (γ-chain specific) peroxidase conjugate (Sigma Chemical Company, St. Louis Missouri, USA). Sigma GAM-HRP, used at 1:5000, was superior because it did not require absorption with *E. ictaluri* WCL to remove cross-reactive antibodies. Antigenic spots and bands were detected using ECL (Amersham International plc, Buckinghamshire England, UK) and blue-light sensitive autoradiography film (Amersham). To aid in spot identification, nitrocellulose membranes were stained with a colloidal gold total protein stain (Bio-Rad) following immunodetection. Autoradiography films of antigenic proteins for each WCL were compared with total protein stained (colloidal gold) nitrocellulose membranes and total protein stained (silver) gels to confirm the identification of antigenic spots and determine their location amongst non-antigenic proteins in total protein stained gels.

Preliminary results indicated variation in spot recognition and intensity between western blots. Therefore, 4 to 10 denatured and 2 to 8 native 2D-PAGE western blots were done for each of the four WCL samples. In four instances, nitrocellulose membranes were stripped and reprobed following the ECL protocol. Each western blot was surveyed for individual antigenic protein spots present. Antigenic spots ranged from light grey to black on autoradiography films.

RESULTS

Total Proteins in 2D-PAGE Gels

All protein bands separated by one-dimensional SDS-PAGE appeared to be resolved into multiple proteins when separated by 2D-PAGE (Figure 2.1). Total protein, silver staining of *E.*

ictaluri proteins in gels showed D2D-PAGE resolved BHI and MM19 grown WCL proteins into at least 245 spots compared to at least 55 bands identified by one-dimensional SDS-PAGE in the sample lane (Figure 2.1). Comparisons of protein spot patterns of total protein stained gels of the WCLs showed no difference in total protein expression between virulent strains 93-146 and 92-266 or at growth temperatures of 25 °C or 30 °C for BHI grown WCLs. The overall spot pattern of the MM19 grown WCL was similar to those of BHI grown WCLs, with the exception of a unique 57 KD protein, with an approximate pI of 6.0 (Figure 2.1). Although there appeared to be differences in the level of protein expression between many proteins, only differences in antigenic proteins, as determined by western blotting described below, are presented. There was some variation from gel to gel, but two antigenic spots had consistently different expression between BHI and MM19 grown WCLs in total protein stain gels based on staining intensity and spot size. A 71 KD spot had higher expression in the MM19 grown WCL than in BHI grown WCLs, and a 58 KD spot had lower expression in MM19 grown WCL than in BHI grown WCLs. These proteins both had approximated pI values between 5.2 and 5.5 (Figures 2.1 and 2.2).

Antigenic Proteins in D2D-PAGE Western Blots

Since detection of the LPS ladder interfered with spot and band identification in western blots probed with CCS that was not LPS absorbed, all blots reported were probed with CCS-LA. Twenty-one D2D-PAGE western blots probed 25 times with CCS-LA were evaluated. Detection varied from blot to blot with some bands and spots being recognized more consistently than others. A total of 39 bands and 89 spots were identified as antigenic proteins in BHI-grown WCLs. Comparisons of western blots probed with CCS-LA showed no difference in expression of antigenic proteins between virulent strains 93-146 and 92-266 and growth temperatures of 25 °C and 30 °C for BHI-grown WCLs. The same antigenic bands and spots recognized in BHI-grown WCLs were detected in the MM19-grown WCL, with the exception of the unique 57 KD spot which was only detected in the MM19 WCL (Figure 2.2B). Total protein stained D2D-PAGE gels showed all of the antigenic bands were resolved into multiple spots, most of which were not antigenic proteins. Three bands of 54, 42 and 10 KD were recognized strongly in most western blots of both BHI and MM19-grown WCLs. Control western blots probed with NCS, the primary

Figure 2.1: Total proteins in silver-stained gels of proteins in whole cell lysates (WCLs) of *Edwardsiella ictaluri* separated by denatured two-dimensional polyacrylamide gel electrophoresis. (A) cells grown at 25 °C in brain-heart infusion broth and (B) cells grown at 25 °C in defined minimal media. Isoelectric points (pI) and molecular weight (in kilodalton, KD) reference points are denoted. Antigenic protein spots that differed in expression between the BHI and MM19 WCLs are labeled by their molecular weights. The 57 KD protein was only expressed in the MM19 WCL.

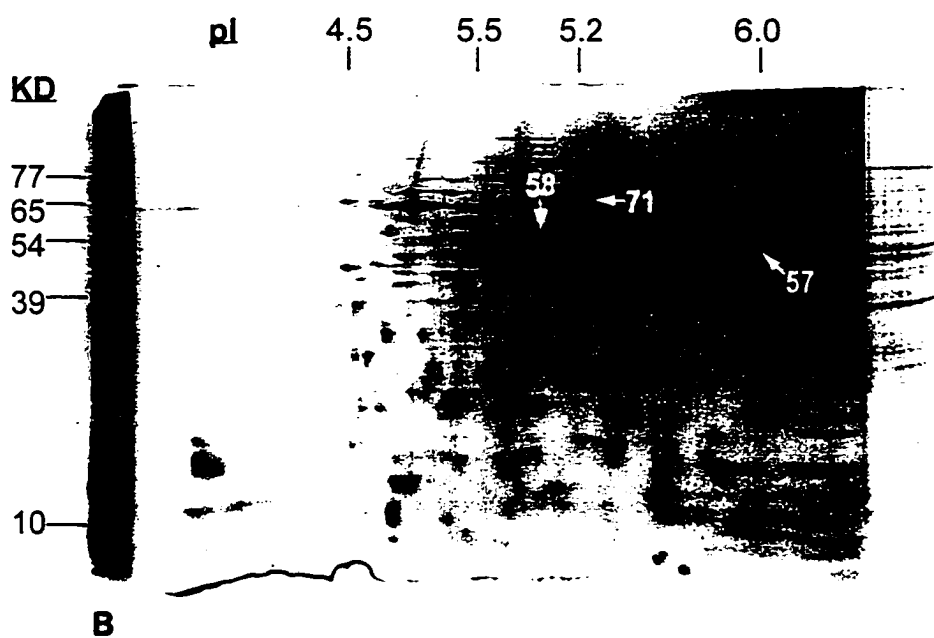
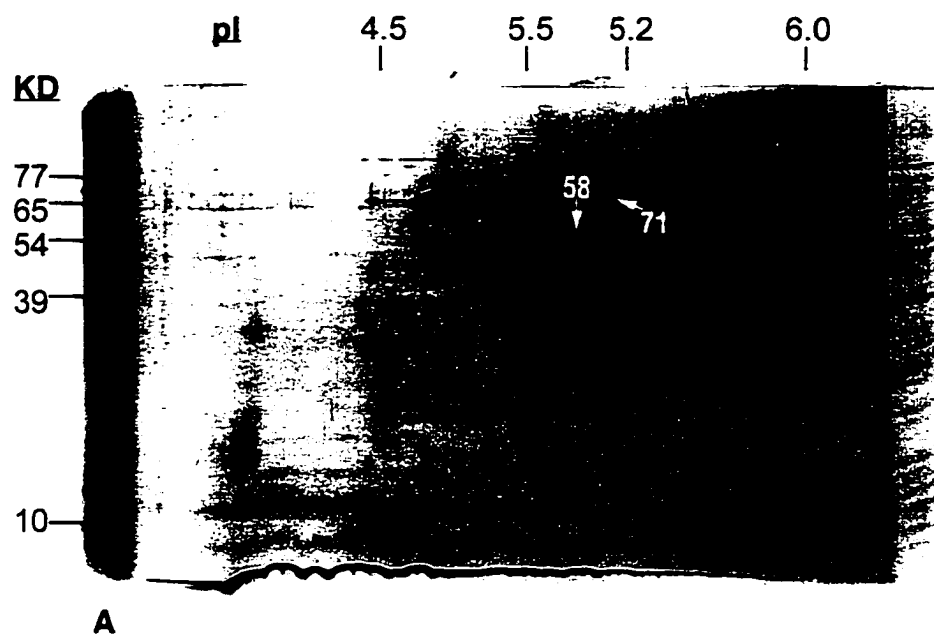
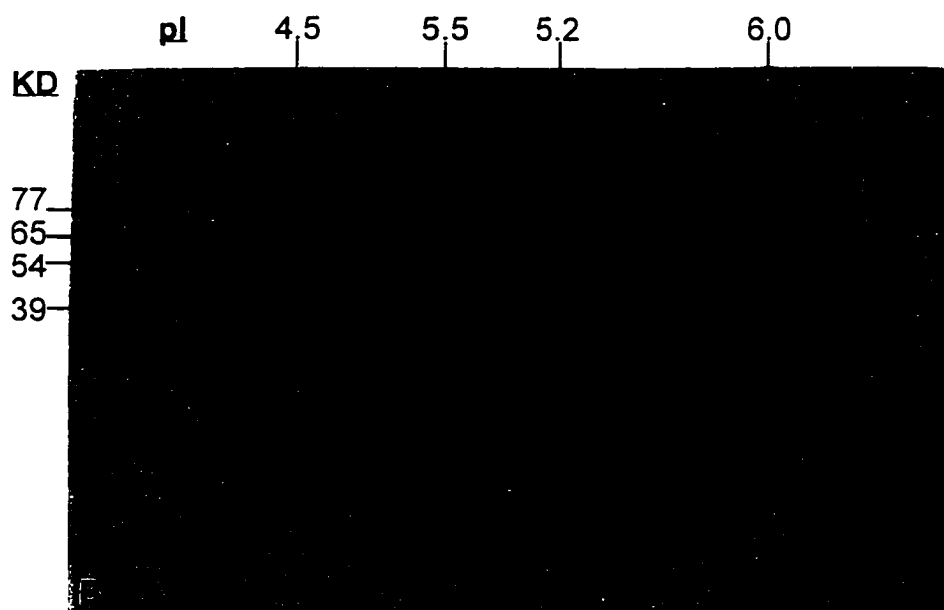
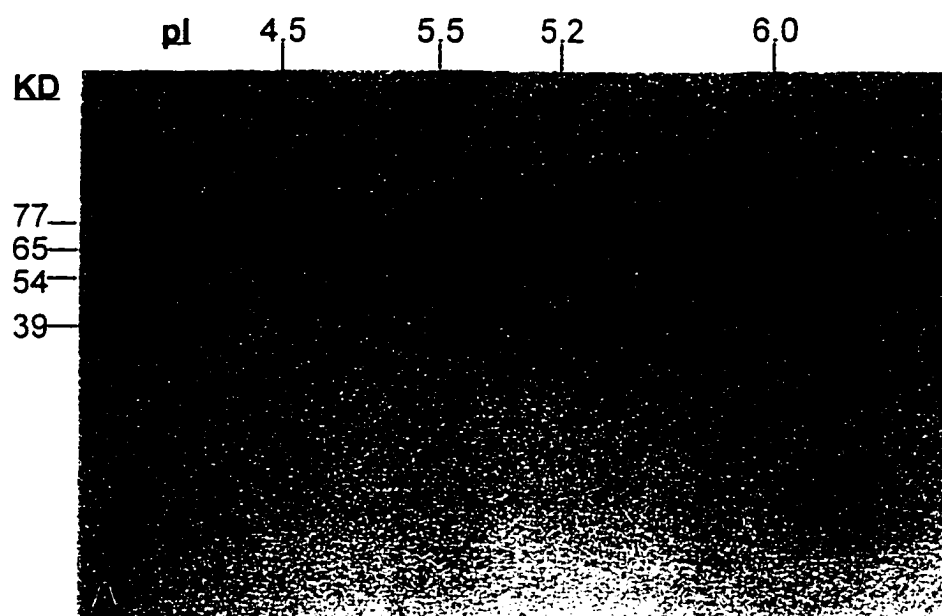


Figure 2.2: Antigenic proteins in whole cell lysates of *Edwardsiella ictaluri* separated by denatured two-dimensional polyacrylamide gel electrophoresis and western blotted with pooled convalescent catfish serum that was lipopolysaccharide-absorbed. (A) cells grown at 25 °C in brain-heart infusion broth and (B) cells grown at 25 °C in defined minimal media. Isoelectric points (pI) and molecular weight (in kilodalton, KD) reference points are denoted. Antigenic protein spots that differed in expression between the BHI and MM19 WCLs are labeled by their molecular weights. The 57 KD protein was only expressed in the MM19 WCL.



antibody, or with secondary and tertiary antibodies alone showed these bands and spots were recognized specifically by CCS-LA. A western blot probed with NCS was run as a control with each set of blots and was consistently negative (data not shown). Absorption of NCS with *E. ictaluri* LPS was not necessary because there were no anti-*E. ictaluri* antibodies in the NCS.

Total Proteins in N2D-PAGE Gels

Most protein bands separated by one-dimensional native PAGE appeared to be resolved into multiple proteins when separated by N2D-PAGE. Silver staining of total *E. ictaluri* proteins in a gel showed N2D-PAGE gels resolved BHI grown WCL proteins into at least 74 spots compared to at least 32 bands when separated by one-dimensional native PAGE in the sample lane (Figure 2.3A). The MM19 grown WCL proteins were resolved into at least 90 spots compared to at least 32 bands (Figure 2.3B). Comparisons of protein spot patterns of total protein stained gels of the WCLs showed no difference in protein expression between virulent strains 93-146 and 92-266 and growth temperatures of 25 °C and 30 °C. Although some areas of the ND2-PAGE gels were similar, the overall spot pattern of the MM19 grown WCL had clear differences in spot patterns and/or level of expression compared to BHI grown WCLs.

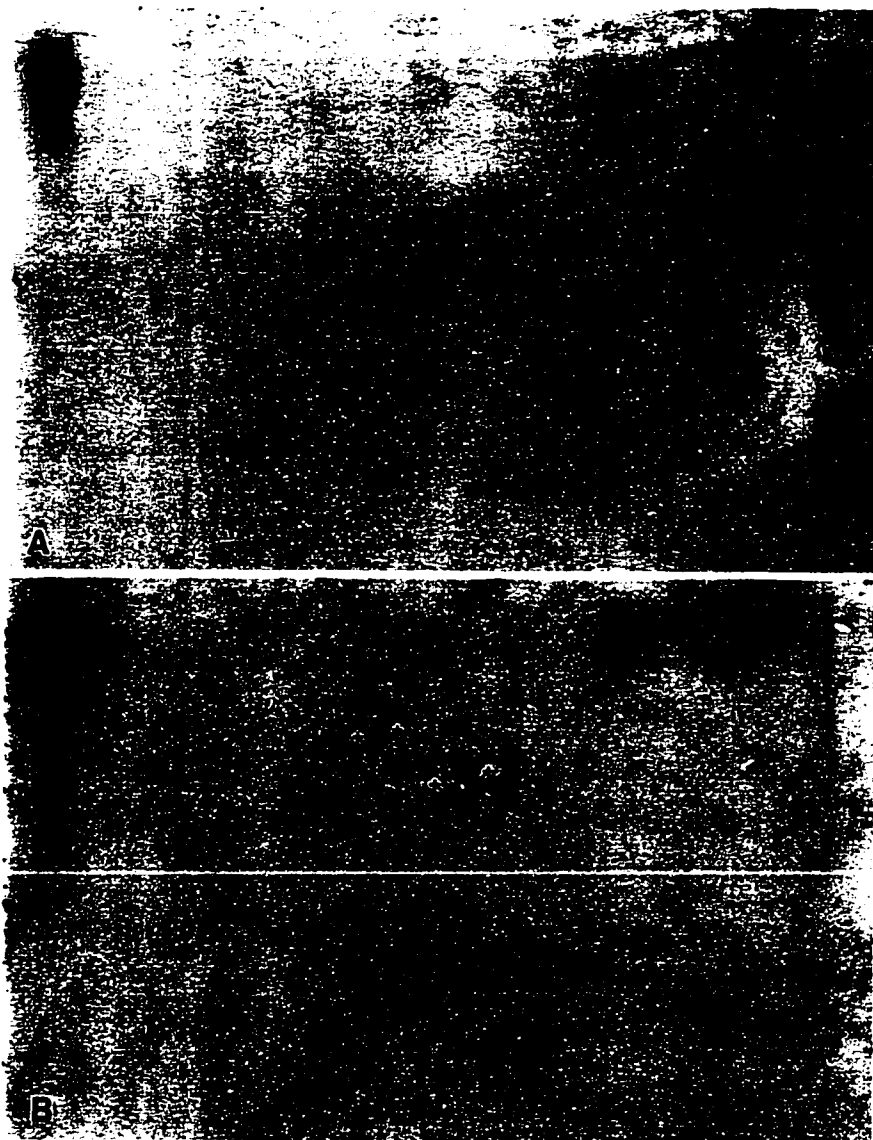
Antigenic Proteins in N2D-PAGE Western Blots

Twenty-two N2D-PAGE western blots probed with CCS or CCS-LA were analyzed. No differences were seen between WCL blots probed with CCS or CCS-LA. A total of five spots were identified as antigenic proteins in BHI WCLs. Comparisons of antigenic proteins identified in western blots of BHI WCLs showed no difference in expression of antigenic proteins between virulent strains 93-146 and 92-266 or at growth temperatures of 25 °C and 30 °C. Although differences in spot patterns between BHI and MM19 grown WCLs made it difficult to make comparisons, the same antigenic spots recognized in BHI WCLs were recognized in the MM19 WCL. However, seven additional spots were also antigenic (Figure 2.4). The additional MM19 grown WCL proteins were not present in BHI grown WCLs based on comparisons of total protein stained N2D-PAGE gels. Control western blots probed with NCS, the primary antibody control, or with secondary and tertiary antibodies alone showed these spots were recognized specifically

Figure 2.3: Total protein, silver stained gels of proteins in whole cell lysates of *Edwardsiella ictaluri* separated by native two-dimensional polyacrylamide gel electrophoresis. (A) cells grown at 25 °C in brain-heart infusion broth and (B) cells grown at 25 °C in defined minimal media. Antigenic protein spots that differed in expression between the BHI and MM19 WCLs are labeled (+; see Figure 2.4).

**A****B**

Figure 2.4: Antigenic proteins in whole cell lysates of *Edwardsiella ictaluri* separated by native two-dimensional polyacrylamide gel electrophoresis and western blotted with pooled convalescent catfish serum that was not lipopolysaccharide-absorbed. (A) cells grown at 25 °C in brain-heart infusion broth and (B) cells grown at 25 °C in defined minimal media. Antigenic protein spots that differed in expression between the BHI and MM19 WCLs are labeled (+).



by CCS and CCS-LA. A western blot probed with NCS was run as a control with each set of blots and was consistently negative (data not shown). Absorption of NCS with *E. ictaluri* LPS was not necessary because there were no anti-*E. ictaluri* antibodies in NCS.

DISCUSSION

Previous studies identified antigens of *E. ictaluri* WCLs using SDS-PAGE and colorimetric western blot analysis with catfish immune serum. Plumb and Klesius (1988) identified immunodominant antigens of 34 and 60 KD using pooled sera from infected and immunized, protected catfish. Subsequently, Klesius and Horst (1991) used a monoclonal antibody (mAb AA224) to identify 36 and 60 KD antigens from *E. ictaluri* localized on the cell surface. Baldwin (1997) used LPS-absorbed pooled convalescent catfish serum to identify 14 antigenic bands out of a total of 38 bands detected by Coomassie brilliant blue staining. Enriched bacterial fractions were used in conjunction with WCLs to aid in identification of some of the antigenic bands. Six of 14 bands were identified as outer membrane proteins, and one was identified as a flagellar protein. Identity of the remaining nine bands is unknown. In WCLs, 39 and 37 KD bands were both recognized strongly by the catfish antiserum, only the 39 KD protein was recognized in preparations of outer membrane proteins. Vinitnantharat and colleagues (1993) conducted vaccination studies using *E. ictaluri* cell extract, crude membrane protein and a purified 36 KD outer membrane protein. Protection was not evaluated, but they showed all were highly immunogenic, though antibody titers were significantly different between the three treatments and lowest with the 36 KD protein.

The present study identified a total of 39 antigenic bands and 90 antigenic spots in BHI and MM19 grown WCLs using one-dimensional SDS-PAGE, D2D-PAGE and ECL western blotting with CCS-LA. Three of the antigenic bands, with molecular weights of 54, 42 and 10 KD, were recognized strongly. One antigenic spot with a molecular weight of 57 KD was expressed exclusively by *E. ictaluri* cells grown in MM19. Total protein stained D2D-PAGE gels showed all of the antigenic bands were resolved into multiple spots, many of which were not antigenic proteins.

The relationship between the 54, 42 and 10 KD bands identified in this study, the 39 KD band identified by Baldwin (1997), and the 34 and 60 KD immunodominant bands identified by Plumb and Klesius (1988) is unknown. However, due to differences in sample preparation, the use of different molecular weight standards, and variation from gel to gel, it is possible that the 60 KD protein identified by Plumb and Klesius (1988) is the same as the 54 KD band identified in this study. The 39 and 34 KD bands identified previously (Baldwin, 1992; Plumb and Klesius, 1988) may correspond with each other and the 42 KD band identified in this study. Western blotting showed mAb AA224 used by Klesius and Horst (1991) did not react with proteins identified in this study. In SDS-PAGE western blots of *E. ictaluri* WCLs, mAb AA224 recognized multiple bands that ran in the pattern of an LPS ladder. No bands were recognized after mAb AA224 was absorbed with purified *E. ictaluri* LPS (data not shown).

Edwardsiella ictaluri LPS is highly antigenic in naturally infected catfish (Baldwin 1997). However, due to the fact that LPS is a T cell independent (TI) antigen which cannot generate an immunological memory response (Arkoosh, et al. 1991), purified LPS is not a protective antigen against ESC unless injections are given in Freund's complete adjuvant (Saeed and Plumb 1986). Baldwin (1992) compared western blots of *E. ictaluri* WCL probed with either LPS-absorbed or non-absorbed, convalescent catfish serum. He found the 39 KD immunodominant protein band was the only band that could be distinguished from the LPS ladder when non-absorbed serum was used. In the present study an LPS ladder was not apparent in total protein stained 2D-PAGE gels. It was recognized faintly in western blots of 2D-PAGE gels using CCS serum that was not LPS absorbed, though protein bands and spots could still be discerned. An LPS ladder was not detected in western blots of 2D-PAGE gels. The difference in LPS detection between this study and Baldwin's was probably due to differences in the amount of LPS present in samples as a result of differences in sample preparation. Baldwin's samples were lysed in SDS with 2ME and frozen. Samples in this study were sonicated in DDI water, centrifuged and supernatant was retained and frozen. Both studies used the same source of purified *E. ictaluri* LPS to absorb serum.

Naturally infected catfish may recognize many of *E. ictaluri* antigens in their native state. Antibodies generated against native proteins may or may not be able to recognize the same proteins in a denatured state. For this reason, it is useful to compare antigenicity between both denatured and native proteins. In the present study the pattern and number of bands and spots differed considerably between silver stained denatured and native 2D-PAGE gels. In contrast, Plumb and Klesius (1988) saw no difference in band patterns between Coomassie brilliant blue stained gels of reduced and non-reduced WCL samples. Plumb and Klesius (1988) ran their non-reduced samples in PAGE gels without detergent, however, 0.5% v/v Nonidet P40 nonionic detergent was used in preparing their WCL. Because nonionic detergent concentrations above 0.1% can result in protein denaturation (Bollag and Edelstein 1991), similarity in reduced and non-reduced samples could be due to denaturation of proteins during WCL preparation.

The present study used denatured and native mini 2D-PAGE with ECL western blotting to show the expression of antigenic and non-antigenic proteins were identical for two virulent isolates of *E. ictaluri*. Previous studies on channel catfish isolates of *E. ictaluri* have also demonstrated a very high homology in protein expression and serology. Bertolini and colleagues (1990) demonstrated protein and LPS homology between 32 isolates of *E. ictaluri* analyzed by SDS-PAGE, and serologic homology between the same isolates analyzed by microagglutination assays using rabbit antiserum. Newton et al. (1990) demonstrated homology of outer membrane proteins between 28 isolates of *E. ictaluri* analyzed by SDS-PAGE, and Plumb and Klesius (1988) demonstrated antigenic homogeneity between 14 isolates of *E. ictaluri* using SDS-PAGE and western blotting with catfish antiserum.

Natural ESC outbreaks generally occur between 22 and 28 °C (MacMillan 1985; Francis-Floyd, et al. 1987), and optimal growth temperatures for *E. ictaluri* cultured in the laboratory are between 25 and 30 °C (Hawke, et al. 1981), but the effect of culture temperature on protein expression of *E. ictaluri* has not been previously evaluated. Plumb and Vinitnantharat (1989) examined biochemical and biophysical characteristics of 40 strains of *E. ictaluri* and found production of gas from glucose occurred in all strains at 25 °C but in only 60% of the strains at

30°C. They also found the organism to be motile at 25-30°C, though motility was reduced at 30°C. Our results show there is no detectable difference in expression of antigenic and non-antigenic proteins for *E. ictaluri* isolate 92-266 grown at 25°C or 30°C. Although induction of heat shock proteins has been widely studied in other organisms, this study did not examine *E. ictaluri* protein expression in culture temperatures above 30°C.

Other researchers have used D2D-PAGE to evaluate differences in protein induction under various stress conditions, showing multiple proteins are repressed or induced under different conditions of growth (Kwaik, et al. 1993; Rafie-Kolpin, et al. 1996). The effects of growth in minimal media has been examined in *Brucella abortus* using D2D-PAGE, with results showing induction of 14 new proteins compared to proteins induced by growth in a rich medium (Rafie-Kolpin, et al. 1996). Previous studies evaluating antigen expression of *E. ictaluri* used enriched culture media such as blood agar, BHI broth, or trypticase soy agar. Our data indicate use of the defined minimal media (MM19) described by Collins and Thune (1996) results in significant differences in expression of antigenic and non-antigenic proteins. A 57 KD antigenic protein, approximated pI 6.0, was only expressed in MM19, and the levels of expression of two antigenic proteins of 71 and 58 KD, pI 5.2-5.5, differed between the two growth media. Significant differences were also apparent in proteins that were not antigenic.

Native 2D-PAGE showed even greater differences between BHI and MM19 WCLs, with about 16 more total spots resolved and seven additional antigenic spots identified in the MM19 WCL compared to BHI WCLs. The D2D-PAGE total protein stained gels showed expression of a single unique protein in the MM19; therefore, the 16 additional spots resolved in N2D-PAGE total protein stained gels of MM19 WCL, with the exception of the unique protein identified by D2D-PAGE, are the same proteins as those detected in BHI. Differences in protein separation between N2D-PAGE gels of MM19 and BHI WCLs may be due to conformational changes in proteins due to growth in different media. Seven additional spots were antigenic in western blots of N2D-PAGE MM19 WCLs compared to BHI WCLs. This suggests growth in MM19 media resulted in protein conformations with a higher number of epitopes exposed for antibody

recognition by CCS. Alternatively, there could be fewer spots recognized in BHI N2D-PAGE western blots because the conformation of proteins resulted in aggregation, with antibodies recognizing multiple proteins and epitopes aggregated as a single spot. Protein aggregation could also mask epitopes of some proteins so they would not be recognized by antibodies in the BHI N2D-PAGE blots.

In conclusion, this research successfully used 2D-PAGE and ECL western blotting to expand the identity of antigenic proteins expressed by *E. ictaluri*. Total protein stained 2D-PAGE gels clearly showed protein bands separated by one-dimensional PAGE are made up of multiple proteins, both antigenic and non-antigenic. Since a single band in a SDS-PAGE gel may consist of more than one protein, gel-purification of a protein from a band will likely result in preparations contaminated by other proteins. Furthermore, LPS may also be present in protein bands even if it is not detected in the total protein stain. Consequently, use of proteins obtained in this manner to generate polyclonal or monoclonal antibodies may result in antibodies against contaminating proteins and/or LPS.

This study also showed culture conditions can affect expression of antigenic *E. ictaluri* proteins. Denatured and native 2D-PAGE and ECL western blotting showed two virulent strains of *E. ictaluri* are antigenically homogeneous and expression of antigenic and non-antigenic proteins are not affected by differences in routinely used culture temperatures, but are affected by culture media. Differences in both protein expression and antigenicity were seen between cells grown in minimal (MM19) and rich (BHI) culture media. The most notable difference is the expression of a unique 57 KD antigenic protein which was only expressed in MM19 media. The function of the unique protein is unknown. Identification of proteins expressed *in vivo* is dependent on the ability of culture medium to mimic the host environment *in vitro* (Mahan, et al. 1993). Although the unique MM19 protein may not be associated with virulence, recognition by CCS-LA demonstrates it is expressed during an *E. ictaluri* infection. This suggests the MM19 media is more analogous to the host environment than BHI, making it a better media for use in studies of *E. ictaluri* antigens and virulence factors.

Finally, the importance of antigenic *E. ictaluri* proteins in the catfish protective immune response is not known. The first step in identifying protective antigens for possible vaccines is to generate candidate antigens by identifying bacterial proteins that are antigenic in the host following infection. This study generated a group of candidates for further study using pooled convalescent catfish serum and 2D-PAGE to resolve the antigenic proteins of *E. ictaluri* from other non-antigenic proteins with the same molecular weights. Protective capabilities of some antigens identified in this research are currently being evaluated in our laboratory.

CHAPTER 3: CLONING AND CHARACTERIZATION OF ANTIGENIC *EDWARDSIELLA ICTALURI* PROTEINS

An *Edwardsiella ictaluri* genomic library in the *Escherichia coli* expression vector XL0LR pBK-CMV (Stratagene) was screened using goat anti-*E. ictaluri* serum (GAI), resulting in isolation of 32 positive clones expressing antigenic *E. ictaluri* proteins. Clone whole cell lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotted with GAI and with pooled convalescent catfish anti-*E. ictaluri* serum (CCS), and plasmid DNA of each clone was analyzed by restriction enzyme digestion. Based on restriction enzyme cutting patterns of the inserts and expression of antigenic cloned proteins in SDS-PAGE western blots, nine clones were selected for further analysis. Partial DNA sequence data was obtained from each clone using primers flanking *E. ictaluri* inserts, and *E. ictaluri* antigenic protein expression was analyzed by two-dimensional (2D)-PAGE and western blotting with GAI and CCS. The DNA inserts of clones pBK-ei4d6 (4d6), pBK-ei5d2 (5d2) and pBK-ei5d3 (5d3) were double-strand sequenced. Clone 4d6 had a unique insert 5727 bp long. Four open-reading frames were identified which appeared to correspond to antigenic proteins expressed by the *E. coli* clone and to three antigenic proteins of 63, 20, and 18 KD expressed by *E. ictaluri*. Genes encoding these proteins did not have sequence homology with any other known sequences. Clones 5d2 and 5d3 had overlapping *E. ictaluri* inserts spanning a region 6362 bp long. The inserts encoded two partial genes homologous with *E. coli* genes *serA* and *pgk*, and five complete genes homologous with *E. coli* genes *rpiA*, *iciA*, *yggE*, *yggB* and *fda*. From encoded and expressed protein results, a cloned antigenic protein of 33 KD was putatively identified as YGGE, encoded by the *yggE* homolog; a 27 KD protein was putatively identified as ribose phosphate isomerase A, encoded by the *rpiA* homolog; a 35 KD protein was putatively identified as the inhibitor of chromosome initiation of replication A protein, encoded by the *iciA* homolog; and a 45 KD protein was putatively identified as fructose 1,6-bisphosphate aldolase, encoded by the *fda* homolog. Since conserved cross-reactive proteins are often T cell antigens, the high antibody cross-reactivity and conservation of proteins between *E. coli* and *E. ictaluri*, as well as other bacteria suggests the cloned antigenic proteins of 5d2 and 5d3 could be T cell antigens.

INTRODUCTION

Edwardsiella ictaluri, the causative agent of enteric septicemia of catfish (ESC), is the primary bacterial pathogen of commercially produced channel catfish (*Ictalurus punctatus*) in the Southeastern United States. Mortality losses and treatment costs are estimated to be 19 million dollars annually (Thune, et al. 1997b). Although ESC is currently treated with antibiotics, increases in the number of antibiotic-resistant *E. ictaluri* strains are reducing the effectiveness of these drugs (Thune and Johnson 1992).

It is generally assumed fish that survive an *E. ictaluri* infection are resistant to ESC if they recover (Klesius and Horst 1991). Vaccination has been shown to be a successful means of disease control for other fish pathogens and *Edwardsiella ictaluri* is considered a good candidate for vaccine development because, unlike most catfish pathogens, it has a very high serologic homogeneity (Plumb 1988; Plumb and Vinitnantharat 1989; Bertolini, et al. 1990). Like other fish pathogens, vaccines against *E. ictaluri* initially focused on using inactivated bacterial preparations. Unfortunately, this approach has been unsuccessful and efforts are now required to define immunity to ESC, including the identification of important antigens.

The role of specific antibodies in protection against ESC has not been determined definitively, but protection has been correlated with antibody titer (Vinitnantharat and Plumb 1993), and antibodies do appear to play a role in the immune response to *E. ictaluri* when combined with phagocytic cells (Scott, et al. 1985; Sheldon and Blazer 1991; Waterstrat, et al. 1991; Wise, et al. 1993; Shoemaker, et al. 1997). Because specific antibodies, activated macrophages and helper T cells may all be components of the protective immune response, a vaccine designed to elicit these responses would theoretically provide protective immunity against ESC. One approach to developing a vaccine of this type is to identify antigens of *E. ictaluri* that elicit the protective humoral and cellular responses.

One approach to identifying protective antigens is to first identify candidate bacterial proteins that are antigenic in the host, that is, proteins that react with host antibodies following

recovery from infection. Although an antibody response does not necessarily correspond with protective immunity, identification of antigenic proteins may provide candidate protective antigens for further studies. The purpose of this research was to first generate a pool of clones expressing antigenic *E. ictaluri* proteins, then characterize the encoded genes and expressed proteins of several selected clones.

MATERIALS AND METHODS

Bacterial Strains, Media and Antibiotics

Escherichia coli strains were grown on Luria-Bertani (LB) or NZY media at 37 °C (Sambrook, et al. 1989). The *E. coli* strains XL1-Blue MRF' and XL0LR (Stratagene, LaJolla California, USA) were maintained in LB media, and F' episomes of both were maintained with tetracycline (Tet, 12.5 µg/ml; Sigma Chemical Co., St. Louis Missouri, USA) selection. The λZAP Express™ phage (Stratagene) were grown in XL1-Blue MRF' on NZY agar plates with NZY top agarose without antibiotic selection. Excised pBK-CMV phagemid (Stratagene) were grown in XL0LR on LB media with kanamycin (Kan, 50 µg/ml; Sigma) selection to maintain plasmids derived from the pBK-CMV phagemid.

Blue-white screening was used to determine the ratio of recombinant to non-recombinant plasmids by spreading 100 µl of 40 mM IPTG (isopropylthio-β-D-galactose; Amresco, Solon, Ohio, USA) and 100 µl of 2% w/v X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside; Amresco) on LB plates. Fusion-protein expression was induced in XL0LR pBK-CMV clones by adding IPTG to 2 hour LB broth cultures at a final concentration of 1 mM IPTG, followed by overnight incubation at 37 °C. *Edwardsiella ictaluri* virulent strain 93-146 was grown on tryptic-soy agar with sheep's blood (TSA II) or in defined minimal media broth (MM19; Collins and Thune 1996) at 25-28 °C.

Bacterial Cell Preparations

Edwardsiella ictaluri and *E. coli* XL1-Blue MRF' cells were harvested by centrifugation at 3800 x gravity for 15 min at 4 °C and washed three times in phosphate buffered saline (PBS),

pH 7.3. Cells were resuspended in sterile distilled deionized (DDI) water to a standardized volume of 0.1 mL of cell pellet per mL and sonicated at 40 W at 50% duty cycle for approximately 8 min, or until cell suspensions changed from milky to clear. After 1 hr at 4 °C, sonicated whole cell lysate (WCL) was centrifuged at 12,000 xg for 30 min at 4 °C, and 10% sodium ethylmercurithiosalicylate (thimerosal; Sigma) was added to the supernatant to achieve a final concentration of 0.01% thimerosal. The supernatant was then divided into aliquots and stored at -80 °C.

Induced XL0LR pBK-CMV cells with *E. ictaluri* inserts were treated in one of three ways. For SDS-PAGE analysis, 500 µL (more if growth was poor) of small-scale induced cultures were aliquoted into sterile microfuge tubes and spun at 16,000 x gravity for 5 min at room temperature. Supernatant was discarded and cell pellets were stored at -20 °C until use. For 2D-PAGE and antiserum absorption, induced cultures were processed as described for *E. ictaluri* and XL1-Blue MRF⁺, but whole cell lysates (WCLs) were not centrifuged following sonication. Rather, 10% thimerosal was added to the lysates to achieve a final concentration of 0.01% thimerosal and WCLs were stored at -20 °C.

Uncentrifuged WCLs were used to absorb cross-reactive antibodies from antisera. Prior to analysis by 2D-PAGE, WCLs were thawed, aliquoted into sterile microfuge tubes and spun at 16,000 x gravity for 10 min at room temperature. The pellet was discarded and the supernatant was aliquoted into fresh tubes and either analyzed or frozen at -20 °C until analysis.

***Edwardsiella ictaluri* Antisera**

Goat *E. ictaluri* Antiserum

Hyperimmune goat antiserum against *E. ictaluri* (GAI) was produced by vaccination of a goat with *E. ictaluri* WCL in Freund's complete adjuvant, followed by three weakly booster injections in Freund's incomplete adjuvant.

Convalescent Catfish Anti-*E. ictaluri* Serum

Catfish anti-*E. ictaluri* serum was obtained from convalescent catfish recovered from natural *E. ictaluri* infections at five catfish farms in Louisiana. Pooled convalescent catfish serum

(CCS) was made up of sera from 15 fish (3 from each farm) with high ELISA and agglutination titers of *E. ictaluri* antibodies. Normal catfish serum (NCS), collected and pooled from catfish hatched and reared in the Specific-Pathogen-Free Laboratory at the School of Veterinary Medicine, LSU, was used as the negative antibody control.

Antibody Absorptions

Antisera absorptions to remove cross-reactive antibodies were accomplished by mixing antiserum with WCLs or *E. ictaluri* lipopolysaccharide and incubating at room temperature for 2 h on a rocker. Absorption mixtures were allowed to sit overnight at 4 °C, then centrifuged at 16,000 x gravity for 5 min at room temperature. Supernatant was transferred to sterile microfuge tubes, brought to .01% thimerosal with 10% stock and stored at 4 °C or -80 °C until use.

Preliminary sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) western blots of XL1-Blue MRF' and XLOLR pBK-CMV WCLs probed with GAI showed a high level of recognition of *E. coli* proteins by GAI serum due to either previous exposure to *E. coli* or to cross-reactivity between *E. ictaluri* and *E. coli* antigens. Band patterns of both *E. coli* WCLs were similar and absorption of GAI with either XL1-Blue MRF' or XLOLR WCL appeared to remove the same amount of cross-reactivity. Since there was no apparent difference in absorption, and a large amount of XL1-Blue MRF' WCL had been prepared, GAI used for screening the *E. ictaluri* library was absorbed with WCL prepared from XL1-Blue MRF' cells. This GAI XL1-Blue MRF' absorbed serum was designated GAI-XA.

The GAI-XA used to probe western blots of *E. ictaluri* was absorbed with *E. ictaluri* LPS purified from strain AL83189 (Baldwin, et al. 1997). The LPS -absorbed GAI-XA was designated GAI-XL. For specific-clone absorption studies, GAI-XL was further absorbed with induced XLOLR pBK-CMV WCL or WCL from a particular induced clone expressing an insert. The specific-clone absorbed GAI-XL was designated SAG.

Screening of *E. ictaluri* Genomic Library

Library Construction and Excision

An *E. ictaluri* genomic library was created in the λ ZAP Express™ expression vector. The library was constructed by partially digesting *E. ictaluri* genomic DNA with *Sau*3A I and cloning it into the *Bam*HI site of the vector. The λ ZAP Express™ phage were grown in XL1-Blue MRF'. *In vivo* mass excision of the library was performed by co-infecting XL1-Blue MRF' with the λ ZAP Express™ phage library and ExAssist helper phage (Stratagene). Excised pBK-CMV phagemid, packaged as filamentous particles, was used to infect XL0LR cells grown on LB-Kan plates. Colonies appearing on the plates contained double-stranded pBK-CMV plasmid. On plates spread with IPTG and X-gal, colonies containing plasmids with *E. ictaluri* inserts appeared white and colonies containing plasmids without inserts appeared blue. The *E. ictaluri* plasmid library in *E. coli* host cells was designated XL0LR pBK-ei.

Plating and Replica Plating the XL0LR pBK-ei Library

Immediately following transfection, XL0LR pBK-ei cells were plated and transferred following the methods of Ausubel et al (1994). Briefly, dilutions containing approximately 2150 colony forming units (CFU) of transfected cells were suspended in LB broth and vacuum filtered onto 110 mm, numbered, circular pieces of nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Eight membranes prepared in this manner were transferred, bacteria side-up, to large LB-Kan plates (150 mm x 15 mm) and incubated inverted at 37 °C overnight.

Each membrane was replica plated onto several other membranes following the methods of Ausubel et al (1994). Replica membranes were transferred to LB-Kan plates spread with IPTG, with or without X-gal to induce protein expression and screen for colonies with inserts. These induced replica membranes were used in library screening with GAI-XA antiserum. The original membranes and their replicas were stored on parafilm-wrapped LB-Kan plates at 4 °C until colonies were picked or the membranes were antibody-screened.

Membrane Processing for Antibody Screening of Colonies

Colony lysis was performed following the chloroform based lysis method of Sambrook et al (1989). Indirect antibody screening was performed using enhanced chemiluminescent (ECL; Amersham International plc, Buckinghamshire, England, UK) western blotting and autoradiography film sensitive to blue light (Amersham) following the manufacturers protocol. Primary antibody was GAI-XA, and secondary antibody was GT-34, a monoclonal anti-goat/sheep IgG (γ -chain specific) horseradish peroxidase conjugate (Sigma).

Rescreening of Positive Colonies

Positive spots on autoradiography film were matched to corresponding viable colonies on either an unprocessed replica membrane or the original membrane. Positive colonies were picked and serially diluted in microfuge tubes containing 100 μ L of LB broth. Fifty μ L of each of three dilutions of each colony were spread-plated on LB-Kan plates (100 mm x 15 mm) and incubated at 37 °C overnight.

For each positive colony, the spread-plate having the dilution with the highest number of countable isolated colonies was replica plated onto nitrocellulose membrane following the methods of Sambrook et al (1989). The replica membrane was transferred to an LB-Kan plate spread with IPTG and X-gal and incubated overnight, then processed and re-screened with GAI-XA antiserum. Positive colonies were picked, cultured, and re-screened until each positive pBK-ei clone was in pure culture in XL0LR host cells. Stocks of each clone in pure culture were prepared and stored in 20% glycerol at -80 °C.

Plasmid Analysis of pBK-ei Clones by Restriction Enzyme Digestion

Small scale (mini) preparations of pBK-CMV and the pBK-ei double-stranded plasmid DNA of each clone were obtained by alkaline lysis (Sambrook, et al. 1989). Large scale plasmid preparations were obtained by alkaline lysis using a Qiagen Plasmid Midi Kit (Qiagen Inc., Chatsworth California, USA). Plasmid preparations were restriction enzyme (New England Biolabs, NEB; Beverly Massachusetts, USA) digested with *Not* I to linearize plasmids, and double-digested with *Not* I and *Pst* I or *Pst* I and *Eco* RI to cut the DNA insert DNA from the

plasmids. Restriction digests were done under the manufacturer's suggested conditions using 10 μ L of plasmid DNA and 1 μ L of each enzyme in a 25 μ L total volume reaction mixture that included the appropriate enzyme buffer and bovine serum albumin (BSA) as needed. Digested plasmid DNA was resolved by agarose gel electrophoresis in 0.6% gels at a constant of 45 volts. Fragment sizes were estimated using a 1 Kb DNA ladder (Gibco BRL) as a size standard (Sambrook, et al. 1989).

DNA Sequencing

Double-stranded phagemid DNA was isolated and purified from clones using the Qiagen Plasmid Midi Kit (Qiagen Inc.). Purified phagemid DNA (500 ng) was mixed with primer DNA (1 μ m) and the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Foster City California, USA), which contained AmpliTaq DNA polymerase FS, and each of the four dideoxy-NTP's labeled with a different fluorescent dye.

Cycle sequencing reactions were performed in a thermocycler with cycle conditions at 96 °C for 30 sec, 50 °C for 15 sec, 60 °C for 4 min for 25 cycles. Sequencing reactions of all four dideoxy-NTP's were performed in the same tube, with the growing chain being simultaneously terminated and labeled with the dye that corresponded to the terminal base. Successive rounds of denaturation, annealing and extension resulted in linear amplification of extension products. Following cycle sequencing, excess unincorporated dye terminators were removed from the extension products using Centri-Sep spin columns (Princeton Separations, Adelphia New Jersey, USA). Column purified sequencing reactions were dried in a vacuum centrifuge and stored at -20 °C until sequencing. Immediately before sequencing, dried sequencing reactions were resuspended in Template Suppression Reagent (Perkin Elmer), then analyzed by capillary electrophoresis using the ABI 310 Genetic Analyzer (Perkin Elmer). Three replicate injections of each reaction were run through the analyzer. Partial sequence data was obtained using primers to the T3 and T7 RNA polymerase sites flanking the insert in the pBK-CMV phagemid vector. Complete double-strand DNA sequence data was obtained from the inserts, starting at

the T3 site on the positive strand and T7 site on the negative strand with subsequent primers produced from the generated sequences.

DNA and Deduced Amino Acid Sequence Analysis

Sequence data was entered into the GCG Wisconsin Sequence Analysis Package™ (Genetics Computer Group, Inc., Madison, WI), where primers were selected and DNA sequences were edited and analyzed. Plus and minus strand sequences obtained from each of clone were assembled into one file using Gel Assemble to identify like and contiguous sequences. Generated sequences were compared to other known sequences in the databases maintained at the National Center for Biotechnology Information (NCBI) in Bethesda, Maryland using the Basic Local Alignment Search Tool (BLAST) in GCG. If BLAST failed to show similarities with known sequences in the data base then a WordSearch was performed. Sequences of genes in the database identified by BLAST or WordSearch were compared with *E. ictaluri* sequences using Gap to determine the percent identity and number of gaps between sequences.

To identify coding regions, double-strand sequence data was analyzed using Map to search for open reading frames (ORFs) and Terminator to search for rho-independent termination sites. Deduced amino acid sequences of ORFs were compared to other known proteins using BLAST. The program PC/Gene (Intelligenetics, Inc., Mountain View California, USA) was used to analyze protein structures of deduced amino acid sequences. Amino acid sequences of proteins in the Swiss Protein data base were analyzed using the Swiss-2D service available through the ExPasy World Wide Web molecular biology server of the Geneva University Hospital and the University of Geneva (<http://www.expasy.hcuge.ch>).

Analysis of Cloned Antigenic *E. ictaluri* Proteins

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 0.75 mm thick, 7 cm x 8 cm slab gels. Gels consisted of a separating gel (12% acrylamide, .0375 M Tris-HCl, pH 8.8, 0.1% SDS) and a stacking gel (4% acrylamide, 0.125 M Tris-HCl, pH

6.8, 0.1% SDS). Combs were used to create wells in the stacking gel. Samples were diluted 1:4 in SDS reducing buffer (.0625 M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2ME, 0.13 % bromophenol blue) and heated to 95 °C for 5 min. For analysis of IPTG-induced and non-induced clone cell pellets, pellets were mixed with 250 µL of SDS reducing buffer and heated. Samples and either broad molecular weight (BMW) standards or pre-stained low molecular weight (PS) standards (Bio-Rad Laboratories, Richmond, California, USA) were loaded into wells of the stacking gel and run at 200 V constant for approximately 42 min. Electrophoresis was stopped when the tracking dye was approximately 5 mm from the bottom of the gel. Following SDS-PAGE, proteins were either stained or western blotted.

Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE)

First dimension isoelectric focusing (IEF) was performed in non-heparinized micro-hematocrit tubes. The IEF tube gels were 6 cm long and consisted of 4% acrylamide with 9.2 M urea, 2.0% Triton® X-100, 1.6% pH 5/7 ampholyte (Bio-Rad) and 0.4% pH 3/10 ampholyte (Bio-Rad). Sonicated, centrifuged WCLs were diluted 1:10 in sterile DDI water then mixed with an equal volume of IEF sample buffer containing 9.5 M urea, 2.0% Triton® X-100, 5% 2ME, 1.6% pH 5/7 ampholyte and 0.4% pH 3/10 ampholyte. Tube gels were loaded with 25 µL of WCL in IEF sample buffer, 5 µL 2D-PAGE standards (Bio-Rad), or both, then covered with 25 µL of overlay buffer (9 M urea, 0.8% pH 5/7 ampholyte, 0.2% pH 3/10 ampholyte, 0.25% bromophenol blue). First dimension IEF was run for 10 min at 500 V constant followed by either 3.5 h at 750 V constant or 16.5 h at 150 V constant.

Second dimension 2D-PAGE was the same as SDS-PAGE except slab gels were 1.0 mm thick. Tube gels were extruded from the micro-hematocrit tubes, laid in the trough of the stacking gel, and overlaid with 100 µL SDS reducing buffer. The reference lane was loaded with 10 µL 1:10 WCL sample or 1:10 low molecular weight (LMW) silver stain standard (Bio-Rad) diluted 1:4 in SDS reducing buffer and heated to 95 °C for 5 min. Following second dimension PAGE, proteins were either silver stained or western blotted.

Total Protein Staining

Total proteins in SDS-PAGE gels were stained with Coomassie blue (Sigma) following the methods of Bollag and Edelstein (1991). Total proteins in 2D-PAGE gels were stained with a rapid silver stain (ICN Radiochemicals, Irvine California, USA) following the ICN protocol, except that gels were soaked in 3.5% sulfosalicylic acid, 11.5% trichloroacetic acid solution for one hour and rinsed in DDI water to remove ampholytes before the standard staining procedures. Following the final development step, gels were soaked in a 0.1% acetic acid solution for 10 min and washed in DDI water to decrease background coloration.

Molecular Weight Estimates of Spots and Bands

Band molecular weights were determined from SDS-PAGE gels loaded with samples and LMW standards in the sample lanes. Molecular weights of spots were determined from 2D-PAGE gels of WCLs with LMW standards in the sample lane. Molecular weights were approximated following the methods of Bollag and Edelstein (1991). Relative mobility factors (Rf) were calculated for each molecular weight standard. The molecular weight of each unknown was determined by fitting the unknown Rf into a linear regression equation established using the known \log_{10} molecular weight and Rf values of the standards.

Identification of Cloned Antigenic Proteins by Western Blot Analysis

Proteins were electrophoretically transferred from unstained gels to 0.45 μ m nitrocellulose membranes at 100 V constant for 1 h in 25 mM Tris, 192 mM glycine and 20% v/v methanol, pH 8.3. Nitrocellulose blots were air-dried and stored in the dark until immunodetection. Indirect antibody screening was performed using ECL with either GAI antiserum, as described previously, or CCS. If CCS was the primary antibody, secondary antibody was - monoclonal anti-catfish IgM (mAb 9E1; Miller et al. 1987) and tertiary antibody was affinity isolated, antigen-specific goat anti-mouse IgG (γ -chain specific) horseradish peroxidase conjugate (Sigma). Antigenic spots and bands were detected following the ECL (Amersham) protocol. To aid in spot and band identification, nitrocellulose membranes were stained with a colloidal gold total protein stain (Bio-Rad) following immunodetection.

Autoradiography films of antigenic proteins in WCLs separated by 2D-PAGE were compared with total protein stained nitrocellulose membranes and total protein stained gels to confirm identification of antigenic spots and determine their location among non-antigenic proteins in total protein stained gels. Antigenic spots ranged from light grey to black on autoradiography films.

Protein Sequencing

Edwardsiella ictaluri WCL was separated by 2D-PAGE and western blotted as described previously, except gels were transferred to Immobilon-P, polyvinylidene fluoride membrane (Millipore Corporation, Bedford Massachusetts, USA) rather than nitrocellulose. The Immobilon-P membranes were air-dried, then Coomassie stained to visualize protein spots. Spots of interest were cut out of four replicate membranes with a scalpel, then transferred to individual sterile microfuge tubes. A piece of blank membrane was also cut out of each membrane to serve as a control for background signals. The samples and controls were submitted to the Louisiana State University Protein Center for automated N-terminal amino acid sequencing. The four replicate spot pieces were pooled as were control pieces to insure an adequate amount of protein was analyzed.

Enzyme Assays

Aldolase Assay

Aldolase (fructose 1,6-bisphosphate aldolase) activity was measured in sonicated WCLs of the control XLOLR pBK-CMV (pBK), and clones XLOLR pBK-ei1.4 (1.4), XLOLR pBK-ei5d2 (5d2) and XLOLR pBK-ei5d3 (5d3) using a commercially available aldolase diagnostic assay (Sigma) following the manufacturer's protocol. The assay used a modification of colorimetric procedures described by Sibley and Lehninger (1949). Enzyme activity was measured as Sigma Units/mL. One Sigma unit of aldolase was defined as being able to split one μM of fructose 1,6-diphosphate (FDP) per minute at 37 °C under the conditions of Sigma procedure 752. One Sigma unit of aldolase was also defined as being equivalent to 1.344 Sibley-Lehninger (S-L) Units, with one S-L unit defined as being able to split 1 mm^3 of FDP per hour at 37 °C.

Differences in WCL protein concentrations were equalized by calculating the enzyme activity as Units/mL/ μ g of protein.

Ribose 5-Phosphate Isomerase Assay

Ribose phosphate isomerase activity was measured in sonicated, centrifuged WCLs of control pBK, and clones 1.4, 5d2 and 5d3 following the colorimetric methods of Hove-Jensen and Maigaard (1993). Each WCL was diluted 1:20 in extract buffer (50 mM potassium phosphate, 1 mM EDTA, 50 mM dithiothreitol, 0.1 mg/ml bovine serum albumin; pH 7.2). A 25 μ l volume of diluted WCL was mixed with 175 μ l of 37 °C reaction buffer to give final concentrations of 5 mM ribose 5-phosphate and 50 mM Tris-HCl (pH 7.5). A 25 μ l volume of extract buffer without WCL was mixed with 175 μ l of reaction buffer served as a blank. After a 10 min incubation at 37°C, 2.0 ml of 66% vol/vol H₂SO₄, 70 μ l of 0.12% carbazole dissolved in absolute ethanol, and 70 μ l of 1.5% wt/vol L-cysteine were added and mixed by vortexing, and samples were incubated for an additional 30 min at 37 °C.

Following incubation, color development was measured at A₅₄₀. The amount of ribulose 5-phosphate (Ru-5-P) produced was estimated from a standard curve generated from known amounts of Ru-5-P (Sigma). Five concentrations of Ru-5-P ranging from 0.464 to 0.0 μ mol were used to generate the standard curve. Optical densities (OD) of the standards at A₅₄₀ ranged from .8714 to 0.0006. Any WCL samples with an OD outside the range of the standard curve were diluted further with blank until they fell within range. The ribulose 5-phosphate isomerase activity was expressed as micromoles of Ru-5-P formed per minute per microgram of WCL proteins (μ mol/min/ μ g).

Protein Assay

Proteins level in pBK, 1.4, 5d2 and 5d3 sonicated, centrifuged WCLs used in the enzyme assays were determined using a commercially available protein assay (BioRad) following the manufacturer's protocol. Bovine serum albumin was used as the protein standard.

RESULTS

Library Screening

Eight LB-Kan plates containing a total of approximately 17,200 CFU of XL0LR pBK-ei were screened with GAI-XA resulting in the entire library being screened approximately 2.2 times. A total of 99 potential positive colonies were picked, of these 73 grew and were rescreened. Thirty-three of the 73 colonies were confirmed positive upon rescreening. Thirty-two confirmed positive colonies were re-isolated and rescreened until they were in pure culture. Stock cultures of each of the 32 isolated clones were prepared and stored at -80 °C. Plasmids of each isolated clone were named and numbered based on the replicate number of the membrane the colony was picked from originally.

Analysis of Cloned pBK-ei Plasmids by Restriction Enzyme Digestion

Clone insert sizes were determined by comparing sizes of the digested pBK-ei plasmids with digested 4.5 Kb pBK-CMV plasmid without insert. Estimated sizes of DNA inserts in pBK-ei isolated from the 32 positive clones ranged from 1.9 to 7.7 Kb (Table 3.1). From the agarose gel results, clones were separated into groups by variations in insert size and restriction enzyme cuts.

Based on combined results of insert sizes and restriction enzyme cutting patterns in DNA agarose gels, and cloned protein band patterns in SDS-PAGE western blots, nine clones were chosen for further analysis (Table 3.1). Large scale purified plasmid DNA preparations were obtained for each selected clone, then digested with *Not* I, and double digested with either *Not* I/*Pst* I or *Pst* I/*Eco* RI to confirm the estimated insert sizes. The nine clones had an average insert size of 4.6 Kb. Sizes of the plasmids of the selected clones and their insert sizes are shown in Table 3.1.

Analysis of Positive Clones by SDS-PAGE and Western Blotting

Induced cells of each positive clone were analyzed SDS-PAGE and ECL western blotting. western blots were screened with GAI-XA and with CCS. Cloned antigenic protein

Table 3.1: Estimated sizes, in kilobases, of DNA inserts in plasmids of *Escherichia coli* XLOLR pBK-ei clones expressing antigenic *Edwardsiella ictaluri* proteins. Clones are grouped by band patterns of expressed antigenic proteins in SDS-PAGE western blots (* denotes clones selected for further analysis).

Clone Name	Plasmid	Insert	Protein Group
*1.1	10.2	5.7	1
1.2	9.2	4.7	2
*1.4	8.3	3.8	3
3.7	9.3	4.8	3
2d1	12.2	7.7	4
2d3	12.2	7.7	4
2d4	12.2	7.7	4
5d5	10.5	6.0	4
*5d8	9.0	4.5	4
8.5	9.3	4.8	4
2d8	10.3	5.8	5
3d5	11.1	6.6	5
*4d6	10.4	5.9	5
3d1	12.2	7.7	6
3d2	9.7	5.2	6
*6d5	8.6	4.1	6
6d9	9.6	5.1	6
4.9	9.2	4.7	7
*5d2	8.3	3.8	7
*5d1	9.0	4.5	8
4d5	12.1	7.6	9
5d9	11.4	6.9	9
5d11	9.4	4.9	9
5d14	12.2	7.7	9
6d7	9.5	5.0	9
4d1	11.7	7.2	10
*5d3	9.0	4.5	10
*5d4	9.3	4.8	11
7d5	6.6	2.1	0
7d8	6.4	1.9	0
7d1	11.7	7.2	0
7d9	11.7	7.2	0

bands were detected in 27 of 32 clones. Catfish antiserum was able to detect most of the same western blot results with both catfish and goat antiserum. Protein band patterns of many of the clones appeared to be expressing the same *E. ictaluri* proteins. Based on western blot results with both catfish and goat antiserum, the 32 clones were organized into 11 groups separated by variations in cloned antigenic protein band patterns (Table 3.1).

DNA Sequencing

Automated DNA sequencing was performed on the nine selected clones. Partial sequence data of each clone was obtained using the T3 and T7 primers, which yielded an average of 360 bp of insert sequence after vector sequence was eliminated. Comparisons of T3 and T7 sequences using the Gel Assemble program of GCG showed several of the clones contained the same, similar or overlapping genomic inserts of *E. ictaluri* DNA. Clones 1.1, 4d6 and 5d4 contained unique *E. ictaluri* DNA inserts. Inserts of clones 5d1, 5d8 and 6d5 appeared to be the same except for slight differences in size, with all three inserts in the same orientation behind the vector promoter. Clones 1.4 and 5d2 appeared to contain the same insert except that the insert in 1.4 was 207 bp longer on the T7 end, and the inserts were in opposite orientations behind the vector's promoter. The insert of clone 5d3 partially overlapped with inserts of clones 1.4 and 5d2.

Analysis of T3 sequence data of each cloned plasmid showed all but two *E. ictaluri* inserts had a stop codon within the first 60 bp in the reading frame of the IPTG-inducible pBK-CMV promoter. The first stop codon in inserts of pBK-ei5d4 and pBK-ei1.4 were at 451 and 300 bp respectively. Locations of stop codons are shown in Table 3.2.

Based on sequence data obtained from T3 and T7 primer reactions and cloned antigenic protein bands detected in SDS-PAGE western blots of induced clones, three clones were selected for complete DNA sequencing. Complete double-strand DNA sequence data was obtained for inserts of clones 4d6, 5d2 and 5d3 starting at the T3 site on the positive strand and T7 site on the negative strand with subsequent primers produced from generated sequences.

Table 3.2: Locations of the first stop codon in frame with the IPTG-inducible promoter of pBK-CMV in *E. ictaluri* inserts of each clone.

Clone	Number of base pairs	Stop Codon
1.1	49	TAA
1.4	300	TGA
4d6	24	TGA
5d1	39	TGA
5d2	54	TAA
5d3	56	TAA
5d4	451	TGA
5d8	39	TGA
6d5	39	TGA

Clone 1.1 T3 and T7 Sequence Data

Sequence data of the *E. ictaluri* genomic DNA insert obtained from T3 and T7 primers for the plasmid of clone 1.1 is shown in Figure 3.1. A schematic diagram of the 1.1 insert in relation to T3 and T7 sequence data is shown in Figure 3.2. The DNA sequences from T3 and T7 primers yielded 402 and 338 bp of insert sequence respectively after vector sequence was eliminated. Sequence comparison using BLAST and WordSearch showed the T3 sequence had similarity with an ORF in *E. coli* K-12 identified as o128 (Blattner, et al. 1997; Accession AE000142). Sequence comparison using WordSearch showed a large region of similarity with the *phoA* gene of *Bacillus subtilis* (Hulett, et al. 1991; Accession BSU02550). Alignment of 1.1 T3 sequence with the *E. coli* region encoding o128 showed 67% identity with 5 gaps between the two sequences. Alignment of 1.1 T3 sequence with the *phoA* gene of *B. subtilis* showed 44% identity with 5 gaps between the two sequences. The product encoded by *E. coli* ORF o128 is unknown. The product of *B. subtilis phoA* is alkaline phosphatase A, an extracellular secreted and cell-bound protein.

Sequence comparison using BLAST identified no sequences similar to the 1.1 T7 sequence. Sequence comparison using WordSearch showed the largest region of similarity with

T3

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1 TTGGGCAACATGCCCCGGGAGCGCCCCGCCAGAGTGTGGTTTTACCCGGTAACGTGGCACA 60
61 GCCCTGAAAATTACGGGTATACTCTCCCGGATAACCTGACTGAGCCAGAAACCAAGATGA 120
121 CAAAACTTACCTTACAAGAGCAGATGCTAAAAGCGGGACTTGTGACCCATAAGAAGATGG 180
181 CCAAAGTCCAGAGAACGGCCAAGAAATCACGGGTTTCAGGCCCGCGAGGCCCGCGAGGCCG 240
241 TAGAGGAAAAAAGCAGGCACAGCTTGAGCGGGATAAGCAGCTGAGTGAACAGCAGAAGC 300
301 AGGCGACCATCGCCAAAGAGATCAAAGCCCAGGTTAAGCAGCTGATTGAAATGAACCGAA 360
361 TCAGCCTGAAAAGAGCGATGTCGATTTTACTTTACAGATGGC 402

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T7

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1 CCGACGCTCGAGATCGTTAAAGAGATCATAGAGCTCGGCAATTGTTCTGCGCATACGGGA 60
61 ACGTAGACCGTTTTTCTGCATCTTCAAGGATAAGAGGAATAGTCCGGCGAGCTCTGGAGAC 120
121 AGCACTGCCAATGGGGATCCCCCGGTCAAGTAACAGCCCTCTTATTTGACAGACTGTAGC 180
181 AGTGCGGTGATTGACAATACGCTGCCTTGCCCCGGTGTAAGCCTGGATATCCTGCTGTTC 240
241 GGGGCTTTTTTGGCGGCACGAACTGCATTGTCGGTTGCATCAGAGCCACTGCGATGGCCTG 300
301 TGCATCATTACCACTATTTTTTTTGCCCGCGGACAAAGG 338

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Figure 3.1: Sequence of the *E. ictaluri* genomic DNA insert in clone 1.1 obtained from the T3 and T7 primers.

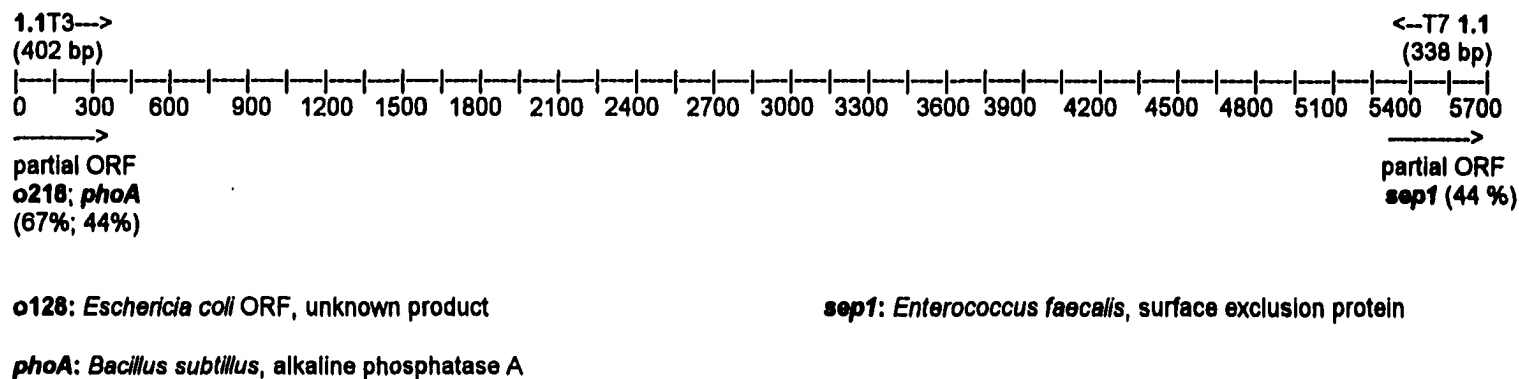


Figure 3.2: Schematic diagram of the insert of *Edwardsiella ictaluri* genomic DNA in clone 1.1 sequenced from the T3 and T7 primers. Sequenced portions are denoted by arrows on top of the insert and putative gene regions based on homologous sequence data is denoted by arrows in the direction they are encoded with the open reading frames (ORF), gene names and percent identity under the inserts. The genes are identified by species and gene products below the diagram. Inserts with regions homologous with more than one gene are separated by a semicolon.

the *sep1* gene in the plasmid DNA of *Enterococcus faecalis* ((Hirt, et al. 1996); Accession X96976). Alignment of the 1.1 T7 sequence with the *sep1* gene of *E. faecalis* showed 44% identity with 6 gaps between the two sequences. The product of *E. faecalis sep1* gene is a surface exclusion protein.

Clone 5d4 T3 and T7 Sequence Data

Sequence data of the *E. ictaluri* genomic DNA insert obtained from T3 and T7 primers for the plasmid of clone 5d4 is shown in Figure 3.3. A schematic diagram of the 5d4 insert in relation to T3 and T7 sequence data is shown in Figure 3.4. The DNA sequences from T3 and T7 primers yielded 451 and 351 bp of insert sequence respectively after vector sequence was eliminated. Sequence comparison using BLAST showed the T3 sequence had similarity with an ORF in *E. coli* K-12 identified as *yicE* (Accession AE000443). Alignment of the 5d4 T3 sequence with *E. coli yicE* showed 76% identity with 2 gaps between the two sequences. The product encoded by *yicE* is a hypothetical 48.9 KD transmembrane, transport protein (Accession P27432) in the xanthine/uracil permease family. The *E. ictaluri yicE* homolog was not in frame with the pBK-CMV IPTG-inducible promoter since the first stop codon, 451 bp inside the insert, fell within the coding region of the *yicE* homolog. Since the partial *yicE* homolog did not have its own promoter, the partial protein encoded by this gene would not be expected to be expressed by clone 5d4.

Sequence comparison using BLAST identified no sequences similar to the T7 sequence. Sequence comparison using WordSearch showed the largest regions of similarity were with an ORF in *Pseudomonas auruginosa* identified as ORF6 (Arai, et al. 1994; Accession D38133), the *rpoN* gene in *Xanthomonas capestris* pv. *vesicatoria* (Accession XCU67179) and the *oac2* gene of *Azorhizobium caulinodans* ((Goethals, et al. 1994); Accession ACOACGNA). Alignment of 5d4 T7 sequence with ORF6 of *P. auruginosa* showed 44% identity with 4 gaps between the two sequences. Alignment of T7 sequence with the *rpoN* gene of *X. capestris* pv. *vesicatoria* showed 44% identity with 3 gaps. Alignment of T7 sequence with the *oac2* gene of *A. caulinodans* showed 43% identity with 5 gaps. The product of ORF6 of *P. auruginosa* is a hypothetical

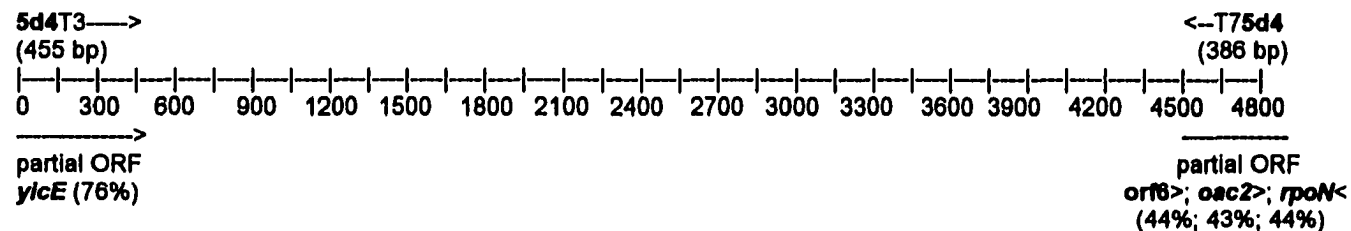
T3

1 AAAACCTGGGGACCTGTTGGCTCCGGTCTGCTGTCGATTCAGGGCACCAGCTTTAACTTT 60
 61 GTCACGCCGCTGATTATGGGCGGCATGACGCTAAAGAACGGCGGCGTCGATATTCCTACC 120
 121 ATGATGGCAACGCTGTTTGGCACCTGATGCTGGCCTCCTGCACCGAAATTATCCTGTCA 180
 181 CGCTTCCTACATCTGGCGCGCTGCATCATCACTCCGCTGGTCTCCGGCATCGTTGTGATG 240
 241 ATCATCGGTCTGTCTCTGATCCAGGTCGGCCTGATCTCCATCGGTGGCGGCTATAGCGCA 300
 301 ATGCACGATCACAGCTTCGGCGCGCCGCAAAAATCTGCTGCTGGCCGGGGCGGTGCTGCT 360
 361 GGTGATCATCCTGCTCAATCGCCAGCGCAATCCCTACCTGCGCGTCGCTCTCTGGTGATC 420
 421 GCGATGGCCGCCGGATATGGACTGGCCTGCTGATG 455

T7

1 TTTCTGAGGAGAATATTATGTATAGAGCAGCACGGATGTATGACTACCGCTGTTGATATG 60
 61 CCCTGTCCACTGCGGCGACATATGCCGCCGCCCTGGCCGATATCCAAACAATGATGGCG 120
 121 CGGGTTGGGGCGGCCAACACGATTTTATCCATAAACTATCGCCAGCCGTTTGTGCTGCGC 180
 181 CAGTGGAATGGATCGCGCCGACGCTATCCTGGCGACGTTTGGCGTTTCCGGCCATGCGTT 240
 241 GATGGATGTTCCGCGACTGTTTTAATCCGCAGGGTCGCATCCCCTTTGTGCTGCCTAACC 300
 360 GGGCGCAGGCGGCCGTCTACCTGTGTCCGGCGCGCTGGGTATTTCCCTGACTCCGCCTTT 400
 401 TCGGGGTTTACGCTATGCTCTCCTTT 486

Figure 3.3: Sequence of the *E. ictaluri* genomic DNA insert in clone 5d4 obtained from the T3 and T7 primers.



yicE: *Escherichia coli*, hypothetical 48.9 KD transmembrane transport protein in the *gltS* (glutamate-specific permease) 3' region

orf6: *Pseudomonas aeruginosa* ORF, unidentified product

oac2: *Azorhizobium caulinodans*, homolog of *Salmonella* dTDP-rhamose synthase

rpoN: *Xanthomonas campestris* pv. *vesicatoria*, transcription factor sigma 54

Figure 3.4: Schematic diagram of the insert of *Edwardsiella ictaluri* genomic DNA in clone 5d4 sequenced from the T3 and T7 primers. Sequenced portions are denoted by arrows on top of the insert and putative gene regions based on homologous sequence data is denoted by arrows in the direction they are encoded with the gene names and percent identity under the inserts. The genes species and gene products are identified below the diagram. Inserts with regions homologous with more than one gene are separated by a semicolon.

protein with an unknown function. The product of the *rpoN* gene of *X. capestri* pv. *vesicatoria* is transcription factor sigma 54. The product of the *oac2* gene of *A. caulinodans* is a homolog of dTDP-rhamose synthase.

Clone 5d1, 5d8 and 6d5 T3 and T7 Sequence Data

The inserts of clones 5d1, 5d8 and 6d5 appeared to be the same except for slight differences in size, with all three inserts in the same orientation behind the vector promoter. Sequence data of the *E. ictaluri* genomic DNA inserts obtained from T3 and T7 primers for the plasmids is shown in Figure 3.5. A schematic diagram of the 5d1, 5d8 and 6d5 inserts in relation to T3 and T7 sequence data is shown in Figure 3.6. The T3 primer yielded 351 bp of insert sequence after vector sequence was eliminated and was identical for all three plasmids. The T7 primers yielded 551 bp of insert sequence after vector sequence was eliminated. The 6d5 T7 sequence went from 1 to 345 bp, 5d1 T7 sequence went from 245 to 549 bp, and 5d8 T7 sequence went from 245 to 597 bp.

Sequence comparison using BLAST showed the T3 sequence had similarity with genes *orf2.8* of *Salmonella enterica* ((Jiang, et al. 1991); Accession X56793) and *galU* of *E. coli* (Weissbom, et al. 1994; M98830) and *Shigella flexneri* (Sandlin, et al. 1995; Accession L32811). Alignment of 5d1, 5d8 and 6d5 T3 sequence with the *S. enterica* region encoding *orf2.8* showed 76% identity with no gaps between the two sequences. Alignment of T3 sequence with the *E. coli* and *S. flexneri* regions encoding *galU* both showed 67% identity with no gaps. The product encoded by *S. enterica orf2.8* and *E. coli* and *S. flexneri galU* genes is α -D-glucosyl-1-phosphate uridylyltransferase. This 32.9 KD protein (Accession P26390) is responsible for O antigen synthesis in the lipopolysaccharide biosynthesis pathway.

Sequence comparison using BLAST identified no sequences similar to 5d1, 5d8 and 6d5 T7 sequence. Sequence comparison using WordSearch showed the largest region of similarity with the *oriC* region in the pIP404 plasmid of *Clostridium perfringens* ((Garnier and Cole 1986); Accession L02938). Alignment of 5d1, 5d8 and 6d5 T7 sequence with the *oriC* region of *C.*

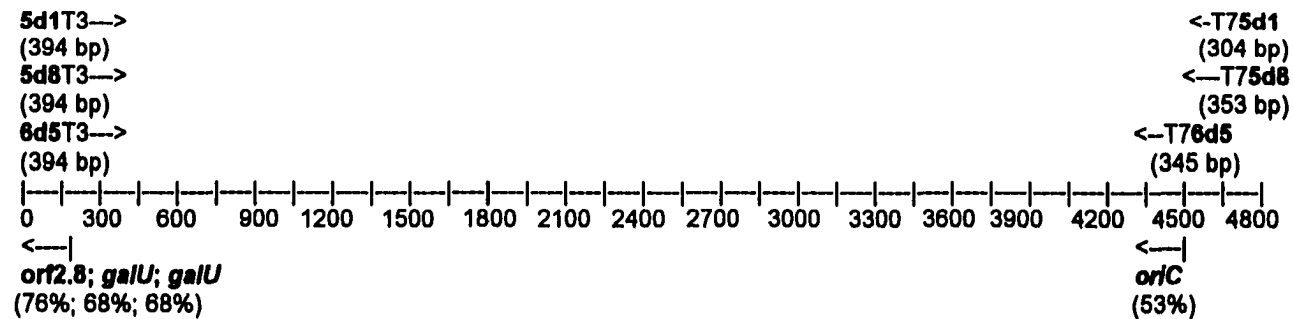
T3

1 TCTTTCACCCCGGCGGACAAACACTCATGGACAACCTTCTGAATCAGCGGCTTATCAACA 60
 61 ATGGGCAGCATCTCTTTCGGGATGGCCTTGGTGGCCGGCAACATCTTCATCCCCAGTCCG 120
 121 GCGACGGGGATAACGGCTTTAAGACTTGTCATCATGCTTCTCCAAAACTCGTAACGAAC 180
 181 CGTATTGGCGATACCCTGCTCTAACGTTACCGGCGCTTTAAATCCCGTTTGCGCGATATG 240
 241 GTTTGACTTAAACTGAGTACGAGCACAAAACCTTCTTCACACGAATGCTGCTTACGGGGAA 300
 301 CTCTCGACCGGTAACTTTGCCAGGATATCGAAGCAATAGCCACCTAACAGGCCAACCGC 360
 361 ATAAGGAATACGAATATTGTTTTTATGGCGATGT 394

T7

1 CTTATTAAGTGTGCTACTTATTTCTTTTGTATTTTCTGTGAGTAATAAGCTTAAAGTGTT 60
 61 AGTTTTATTATTAATGCTTTTTGTGTTTATTTGACAAAATCAAGAACGGCAATATTGGC 120
 121 TTTGGCTGCATTTTTTCTATGTTGTAAGATTAAGCGGAAATTATTGTTGTTCTATTTTAT 180
 181 ATTATTTGTGTCTATAGCCTCTCAGTATTTCTTTTCATACATATTCCCTTCAATACTGTC 240
 241 TGATCTCGATTTTCGGCCATAGCCGATTTTTTGATTTAAATGATGCCAGTAATATGGAAAG 300
 301 GATAACTATTTATAAGGAGGCCGTGGATTTTATATTATCCCATTTTACTCACTTTATTAT 360
 361 TAATGGGGTGACTAATTATTTAGAAATAACAAGTTGGCTACTAATATACCACATAATTG 420
 421 GTTTATCCAGTTTATCTTGGGTTTGGGTTTTTATTTTCTATTTATTATTGCGGGTGGTT 480
 481 GTTTTTGTTGCAAAAATAGTTTCTGGTAAGTCTTATTATCTATTGGCTTTTATGTGTTTT 540
 541 TTCTTTGTTTATGCATCATTTCTCAGTTATTATCCTTTAACAACCTCCATTATTATTT 597

Figure 3.5: Sequence of the overlapping *E. ictaluri* genomic DNA inserts in the clones 5d1, 5d8 and 6d5 obtained from the T3 and T7 primers.



orf2.8; galU; galU: *Salmonella enterica*; *Escherichia coli*; *Shigella flexneri*, α -D-glucosyl-1-phosphate uridylyltransferase.
A 32.9 KD protein responsible for O antigen synthesis in the lipopolysaccharide biosynthesis pathway.

oriC: *Clostridium perfringens* plasmid pIP404 origin of replication

Figure 3.6: Schematic diagram of the inserts of *E. ictaluri* genomic DNA in the clones 5d1, 5d8 and 6d5 sequenced from the T3 and T7 primers. Sequenced portions are denoted by arrows on top of the insert and putative gene regions based on homologous sequence data is denoted by arrows in the direction they are encoded with the gene names and percent identity under the inserts. The genes, species and gene products are identified below the diagram. Inserts with regions homologous with more than one gene are separated by a semicolon.

perfringens showed 53% identity with 1 gap between the two sequences. The oriC region is the origin of replication for the *C. perfringens* pIP404 plasmid.

Clone 4d6 Double-Strand Sequence Data

The double-strand sequence data of the *E. ictaluri* genomic DNA insert of clone 4d6 is shown in Figure 3.7. A schematic diagram of the 4d6 insert in relation to the double-strand sequence data is shown in Figure 3.8. The insert size was determined to be 5727 bp after vector sequence was eliminated. Sequence comparison using BLAST and WordSearch showed no homology with other known DNA sequences. Map identified eleven potential ORFs in the six different reading frames with deduced amino acid sequences encoding proteins of 10 KD or larger. Sequence comparison using BLAST showed no homology of the deduced amino acid sequences with other known proteins. PC/Gene was used to determine molecular weight and pi of the putative protein encoded by each reading frame. Results of the analysis of ORFs are shown in table 3.3. The initial designation of the ORFs was ORF 1 through 11.

Based on combined results of DNA sequence data and antigenic protein expression data of clone 4d6, only four of the eleven putative ORFs encoded proteins with characteristics comparable to the three antigenic proteins that were expressed by the clone. Those results are presented below in the section entitled "Putative Identification of Genes Encoding Antigenic *E. ictaluri* Proteins". Putative promoter regions, identified manually, could be found for nine of the eleven ORFs. The four ORFs were re-named based on the molecular weight of the putative proteins they expressed. Putative transcriptional promoter regions of the four re-named ORFs are shown in Table 3.4 compared to the *E. coli* σ^{70} promoter consensus sequence (Lewin 1990). A promoter region could be identified for ORF eip18, but the region between the purine rich Shine-Delgamo translational ribosome binding sequence and the transcription initiation site was only 2 bp. The eip18 gene may be in an operon with the eip55 gene under control of the eip55 promoter since the genes are in the same reading frame 70 bp apart. A putative rho-independent terminator was also identified 106 bp after the eip55 stop codon.

```

1  CCGGCGCTTCCCTGCTGTTGTACTGACAGGCTGGTTTGTGCGCGGGGGAGAAGCAGGCA 60
   GGCCGCGAAGGGACGACAACATGACTGTCCGACCAAACAGCGCGCCCCCTCTTCGTCCGT

61  TGGATGACTGACACCGGCCAAATTTCCACCGGGTAGGTGGTCCGGAACCGGCATGCCATG 120
   ACCTACTGACTGTGGCCGGTTTAAAGGTGGCCCATCCACCAGGCCTTGCCCGTACGGTAC

121  CCATCCACCTGGCGGGATAGCAGGCTGGTATGTCGTGGCAGCCGAGCGGGCCTGACAAG 180
   GGTAGGTGGACCGCCCTATCGTCCGACCATAACAGCACCGTCGGCGTCGCCCCGACTGTTT

181  TTGGTCTGTGCCGGATCGACCGAAAACCTGGACCAGAGATATGGAGGGAATCGGGCGCAGG 240
   AACCAGACACGGCCTAGCTGGCTTTTGACCTGGTCTCTATACCTCCCTTAGCCCCGCGTCC

241  TAATGGGGATAGAGGATCCCCAGCAACGCATTGGTTATTTCCGGGGAACCTGCTCATCTATC 300
   ATTACCCCTATCTCCTAGGGGTCGTTGCGTAACCAATAAAGCCCCCTTGACGAGTAGATAG

301  TTGTGATGCACGCGGGCGCAGAGAAATGCCACGGCTTCGATCAGCCGCTCGACATGAGGA 360
   AACACTACGTGCGCCCGCTCTCTTTACGGTGCCGAAGCTAGTCGGCGAGCTGTACTCCT

361  TCTTCCGATACTCCCTCCTCCAGCAGAAGGCGGCCGGCTACCTTGGGATACTGGGAGGCG 420
   AGAAGGCTATGAGGGAGGAGGTCTCTTCCGCCGGCCGATGGAACCCCTATGACCCCTCCGC

421  AACTCACGTCCCAACTGGCGGAGGAACGCCAGTTCCCGGTTATAGTAGGGCAATAACTCG 480
   TTGAGTGCAGGGTTGACCGCCTCCTTGCGGTCAAGGGCCAATATCATCCCGTTATTGAGC

481  GCCATGCATCACCTGTCTATTTCGGTACAGCTGTGTTGAAACATCCAGAACGGCATCAAAT 540
   CGGTACGTAGTGGACAGATAAGCCATGTCGACACAACCTTGTAGGTCTTGCCGTAGTTTA
e      * R D I R Y L Q T S V D L V A D F -

541  ACCACCGGCTCTGGAGAGGGCTCCACGTCCAGTAATGCCTCGATCCTGAATATCATCTGG 600
   TGGTGGCCGAGACCTCTCCCGAGGTGCAGGTCAATTACGGAGCTAGGACTTATAGTAGACC
e      V V P E P S P E V D L L A E I R F I M Q -

601  CTGATGCCGGTTTTCTCATCCAGATGGATTTGAACCCGGACCTGACGTAGCCGGGGTTCA 660
   GACTACGGCCAAAAGAGTAGGTCTACCTAAACTTGGCGCTGGACTGCATCGGCCCAAGT
e      S I G T K E D L H I Q V R V Q R L R P E -

661  AACAGGTCAATCACTCGTTCGATATGCTGGCGGATCCACTCCCGATCCTCGGCATTGGCG 720
   TTGTCCAGTTAGTGAGCAAGCTATACGACCGCCTAGGTGAGGGCTAGGAGCCGTAACCGC
e      F L D I V R E I H Q R I W E R D E A N A -

721  GCACTCAGGTTTGTAGCAGTCCGGCAGGCCGTAATTCAGGGGGGATTTTTCCAGCTCAGGC 780
   CGTGAGTCCAAACTCGTCAGGCCGTCCGGCATTAAGTCCCCCCTAAAAGGTGAGTCCG
e      A S L N S C D P L G Y N L P S K E L E P -

781  CACTGGCTTATATCCAGTGCCAGCGCGGATCGCGAATTCAGAATATCTTCCACATCCCGT 840
   GTGACCGAATATAGGTACAGGTGCGGCCTAGCGCTTAAGTCTTATAGAAGGTGTAGGGCA
e      W Q S I D L A L A S R S N L I D E V D R -

841  CTGACGGTCCAGCGAAAATGGGCCAGTTTCATACCGCGGATCTGATGTCCGTTCTGTTCCC 900
   GACTGCCAGGTCGCTTTTACCCGGTCAAGTATGGCCGCTAGACTACAGGCAAGACAAGGG
e      R V T W R F H A L E Y R R D S T R E T G -

901  TCCTGCGGGAAATCATCGAACAACGATCCAGCACGGAAGGGCGGAAGATACGATGAAGG 960
   AGGACGCCCTTTAGTAGCTTGTGTTGCTAGGTCGTGCCTTCCCGCCTTCTATGCTACTTCC
e      E Q P F D D F L R D L V S P R F I R H L -

```

Figure 3.7: Double-strand sequence of the *Edwardsiella ictaluri* insert in clone 4d6 with the deduced amino acid sequences of putative open reading frames encoding antigenic proteins (d, e and f denote reading frames).

(Figure 3.7 con'd)

e

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961 ATCATGGCTCAACTCACGGGATTGTCTTTTTTAGGTTATCGACGCCGTCGGTCGACAGCA 1020
    TAGTACCGAGTTGAGTGCCCTAACAGAAAAATCCAATAGCTGCGGCAGCCAGCTGTCTGT
    I M -
1021 TGA CTGCCAGCGTTTTTCAGATACCCGTCTTTGGTTCCGTGCTCTCCCATCAGCAGTGCGA 1080
    ACTGACGGTCGCAAAAGTCTATGGGCAGAAACCAAGGCACGAGAGGGTAGTCGTACGCT
1081 TGGTGGCGATGGCGGTGCGGCAGCCCGGAGTGGTGACCTGAAAGCCTGGCATAACAGGGCT 1140
    ACCACCGCTACCGCCAGCCCGTCGGGCCTCACCCTGGACTTTTCGGACCGTATGTCCCGA
1141 TTCCGTTTCAGCGGTGGCGCCCCGGGAGTTCCTTTCATCTGGGTTCCCCGGCTTCTGGCAG 1200
    AAGGCAAGTCGCCACCGCGGGGCCCTCAAGGAAAGTAGACCAAGGGGGCCGAAGACCGTC
1201 CCTGAATCATCTGTAAACACATCTGGTGTTTGTGGGAGGCGGTGCCTTGCTGTGGTGGT 1260
    GGACTTAGTAGACATTTGTGTAGACCACAAACACCCTCCGCCACGGAACGGACACCACCA
1261 GCAGCTTGCGTATCCCCATCGCCATCATCGGGAGCAGTCCCACCATGATAAAGCCAGCGG 1320
    CGTCGAACGCATAGGGGTAGCGGTAGTAGCCCTCGTCAGGGTGGTACTATTTTCGGTCGCC
1321 CAAAGCCCACCGCTTTGGCCATATTGATTTGATCATAGCGGTGATAATAAACTTGAGCC 1380
    GTTTTCGGGTGGCGAAACCGGTATAACTAACTAGTATCGCCAGCTATTATTTGAACTCGG
1381 GTCCATCGCACTGACCGCAGGTACAGGGGTACAGATGCTGGCGCAGATCCCACAGCGTGC 1440
    CAGGTAGCGTGACTGGCGTCCATGTCCCATGTCTACGACCGCGTCTAGGGTGTTCGCACG
1441 CATAACATCCATGGTCGAGGCCACGCTGGCCGTGGTCACGGCCAGGTTTCAGTATGACCC 1500
    GTATTTGTAGGTACCAGCTCCGGTGCAGCCGCCACCAAGTGCCGGTCCAAGTCATACTGGG
1501 CGACCGGAGTGGCGGCAGCCGACAGCGCCGTACCGACACCGCCGCCACGGCCTGGCCAA 1560
    GCTGGCCTCACCGCCGTCGGCTGTGCGGCATGGCTGTGGCGGGGGTGGCGGACCGGTT
1561 CGGTGCTCAGGAGCGCATTGCCACCGGCGGAGACACTCCCCTGGGTGGCATTGATAATCT 1620
    GCCACGAGTCCCTCGCGTAACGGTGGCCGCCCTCTGTGAGGGGACCCACCGTAACATTAGA
1621 GCTGGGTGAGCGCAACGTAGGGAGAGACCCCTCCGCAATATTGTTACCGCCGTCAGTG 1680
    CGACCCAGTCGCGTTGCATCCCTCTCTGGGGGAGGCGTTATAACAAGTGGCGGCAGTCAC
1681 CCGTTTTGGCCGGTCTGGGTAATAGAGAACCGGCGGTGTTGATCAGCCCCACGGTACCCA 1740
    GGCAAAACCGGCCAGACCCATTATCTCTTGCCGCCACAACCTAGTCGGGGTGCCATGGGT
1741 GCACATTGGATACCGTTGATGCAGAAAAGGATGCGATGCCCGCTGCGTCTATCGGTAACG 1800
    CGTGTAACCTATGGCAACTACGTCTTTTCTACGCTACGGGGCACGCAGATAGCCATTGC
1801 CCCCAGATGCCGACTTGATATAGCTGGCCAGTGAAGCCTCCTGGTTGGCTCTGTTGATCG 1860
    GGGGTCTACGGCTGAACATATATCGACCGGTCACTTCGGAGGACCAACCGAGACAACCTAGC
1861 CTGTACCGATGTCGCCTATTATCTGCGGGACGTTGATACTATGTCCCCCGCATCAGCAG 1920
    GACATGGCTACAGCGGATAATAGACGCCCTGCAACTATGATACAGGGGGCGTAGTCGTC
1921 GATGACTATCGATAGGCCACTGAGGCGGACGTAGCAATGAGGTGGATTTTTGCTTCATCA 1980
    CTACTGATAGCTATCCGGTGACTCCGCCCTGCATCGTTACTCCACCTAAAAACGAAGTAGT
1981 CATCATCTAGCGATTCTGTAGCATCTTCAACAGCGCGCCAAAGGATGTTGAATCGAACT 2040
    GTAGTAGATCGCTAAGGACATCGTAGAAGTTGTGCGCGGGTTTCTTACAACCTTAGCTTGA
2041 TCAGCTCATGACTGATGACAACATCCGCCATGCGTAGTACCTCGATAGGCATCACCGCCG 2100
    AGTCGAGTACTGACTACTGTTGTAGCGGTACGCATCATGGAGCTATCCGTAGTGGCGGC
2101 CACTGTAAACCCGGCTTGCCGCTTTGGCTTTGGGCCAGTTGTTGCCACCGCTTGTCAT 2160
    GTGACATTTTGGGCCGAACGGCGAAACCGAAACCCGGGTCAACAACGGTGGCGAACAGTA

```

(Figure 3.7 con'd)

8	2161	CATCAGTGAGATCGTCAATATTCATTATGTGCTCCTGCCGATGTAGCCTATCTGTATAAA	2220
		GTAGTCACTCTAGCAGTTATAAGTAATACACGAGGACGGCTACATCGGATAGACATATTT	
	2221	GCGGAAGGGGTATCGCCTATCTGTGTAAAGAGGGGAACGGTACAGGGTGACaTATTTGCG	2280
		CGCCTTCCCCATAGCGGATAGACACATTTCTCCCCTTGCCATGTCCCCTGtATAAACGC	
	2281	ACGCCACCTGTACCTGACCCCCAGCTTGATGGTTTTACTTCTTCTGGTTGGTAATAAGAC	2340
f		TGCGGTGGACATGGACTGGGGGTCGAAC TACCAAAATGAAGAAGACCAACCATTATTCTG	
		* K K Q N T I L -	
	2341	TCCAGCCCTCGGGCCCAATTTTACTGCCCGCCTTACCGCTCTGATCCTGGGGAATATATT	2400
f		AGGTGCGGAGCCCGGGTTAAAATGACGGGCGGAATGGCGAGACTAGGACCCCTTATATAA	
		S W G E P G I K S G A K G S Q D Q P I Y -	
	2401	CCCAAGTCACTTCC TTATAGGCGAAATCAATGGTTTCCTGCGGGCGGTCGCTTCCGCCAG	2460
f		GGGTTCAGTGAAGGAATATCCGCTTTAGTTACCAAAGGACGCCCGCCAGCAAGGCGGTC	
		E W T V E K Y A F D I T E Q P R D S G G -	
	2461	AGGCCCCGGAGCAGTCATAGCTGGAGATGGCTACATTAGTAAATTTATAGATACAGAAGG	2520
f		TCCGGGGCCTCGTCAGTATCGACCTCTACCGATGTAATCATT TAAATATCTATGTTCC	
		S A G S C D Y S S I A V N T F K Y I C F -	
	2521	CGAACTTATCGCCAGCCTGGGTACAAATCTCCACTTCCAGCGAGGGAAAGTTCTGGCCCT	2580
f		GCTTGAATAGCGGTCGGACCCATGTTTAGAGGTGAAGGTCGCTCCCTTTCAAGACCGGA	
		A F K D G A Q T C I E V E L S P F N Q G -	
	2581	GAACGGCGGGCCGAGGCCAGCTTGACACTGGCGCAGTCCATCCATTTGGTTATACGGAATA	2640
f		CTTGCCCGCGGCTCCGGTCGAACTGTGACCGCGTCAGGTAGGTAAACCAATATGCCTTAT	
		Q V A A S A L K V S A C D M W K T I R F -	
	2641	CCGACATTGTGGCCTTACCGGTATT CAGACCCTGGGTACCGCTCTCCATTGAGCACTCAT	2700
f		GGCTGTAACACCGGAATGGCCATAAGTCTGGGACCCATGGCGAGAGGTAAC TCGTAGTA	
		V S M T A K G T N L G Q T G S E M S C E -	
	2701	TGCCTGCTCCCCATGCAAAGCCAGTACTTCAATCCATTTTTTGTGCTTAGCATCAGTAG	2706
f		ACGGACGAGGGGTACGTTTCGGGT CATGAAGTAGGTAAAAAACACGAATCGTAGTCATC	
		N G A G W A F G L V E I W K K H K A D T -	
	2761	ATTCCCCATCAACCTTATCTAGTTTGATATAGGTATCAAAAGCCATAGCGGACCTCTCTT	2820
f		TAAGGGGTAGTTGGAATAGATCAAAC TATATCCATAGTTTTTCGGTATCGCCTGGAGAGAA	
		S E G D V K D L K I Y T D F A M -	
	2821	GTGACAAGATAAACTGTGTTTTAGATATATGTGATAGATAAATTCGGCTAAATAGTTAGC	2880
f		CACTGTTCTATTTGACACAAAATCTATATACACTATCTATTTAAGCCGATTTATCAATCG	
		* -	
	2881	CTTGCGCCGGTGGCGGCAGATCGGCGACCAAACGTAAAGATGCGGTCAGCCCTCCAGCT	2940
f		GAACGCGGCCACCGCCGCTAGCCGCTGGTTTGCATTTCTACGCCAGTCGGGGAGGTGCA	
		G Q A P P P L D A V L R L S A T L G E L -	
	2941	GGAAATGGGGTTTTAAATAGGCCACTGCCTTATAGAACCCCGGTTTTACCTGGAATATCGC	3000
f		CCTTTACCCCAAATTTATCCGGTGACGGAATATCTTGGGGCCAAATGGACCTTATAGCG	
		Q F H P K L Y A V A K Y F G P K G P I D -	
	3001	TGACATCAATACGGGCTTCCCGTAATGGGTATTTTCGCCTTAATATCCTGGCCTGCATCAT	3060
f		ACTGTAGTTATGCCGAAGGGCATTACCCATAAAGCGGAATTATAGGACCGGACGTAGTA	
		S V D I R A E R L P Y K A K I D Q G A D -	
	3061	CTGAACCGACCACATAATTACTGATCCACTGATTGAGAAAGTATTCACATTTCGTACGGC	3121
f		GACTTGGCTGGTGTTAATGACTAGGTGACTAACTCTTTCATAAGTGTAAGCGATGCCG	
		D S G V V Y N S I W Q N L F Y E C E S R -	

(Figure 3.7 con'd)

3121 TCATAAAGCTGCCGAGCTTGTGCGGGACCATCGATTTTAAATAATGGGCAAAGCGCGAGG 3180
 AGTATTTTCGACGGCTCGAACAGCGCTGGTAGCTAAAATTTATTACCCGTTTCGCGCTCC
 f S M F S G L K D R V M S K L Y H A F R S -
 3181 TGGCCATAATATATTGCAGCTGGCAGGCGATCCTGGCGTTGGCATTAGCTGAGTCACTGT 3240
 ACCGGTATTATATAACGTCGACCGTCCGCTAGGACCGCAACCGTAATCGACTCAGTGACA
 f T A M I Y Q L Q C A I R A N A N A S D S -
 3241 CATATTCCTTGGGCTTTTGTGCCGACTGGGTGCGAAAAGAATGCGGCATAGTCGGTACCCT 3300
 GTATAAGGAACCCGAAAACACGGCTGACCCAGCTTTTCTTACGCCGTATCAGCCATGGGA
 f D Y E K P K Q A S Q T S F F A A Y D T G -
 3301 TACAGTGGGTGAGGGCGATAAATCCCAGGTGCGCCAACTCTTTCTCCCGGCGATCGGTAA 3360
 ATGTCACCCAGTCCCGCTATTTAGGGTCCAGCCGGTTGAGAAAGAGGGCCGCTAGCCATT
 f K C H T L A I F G L D A L E K E R R D T -
 3361 TGGCCACTTCGGTGGGACACTTGAGCTCTACTTCGCCATCATCGGTAGTAAAGGTGTGCG 3420
 ACCGGTGAAGCCACCCTGTGAAGTTCGAGATGAAGCGGTAGTAGCCATCATTTCCACACGC
 f I A V E T P C K L E V E G D D T T F T H -
 3421 CCGGCAGTCCCTCCACCAGACCACCTCCTTCGACTCCCCGGATGGCGGCGCACCAGCCAT 3480
 GGCCGTGAGGGAGGTGGTCTGGTGGAGGAAGCTGAGGGGCCATCCGCCGCGTGGTTCGGTA
 f A P L G E V L G G G E V G R I A A C W G -
 3481 ATTTGGCATAGGCCTGAGTCAGGCGGGTACCCAGCGCATAGGCCGCATTGAGCCACAGAT 3540
 TAAACCGTATCCGGAAGTCCGCGCCATGGGTGCGGTATCCGGCGTAAGTCGGTGTCTA
 f Y K A Y A Q T L R T G L A Y A A N L W L -
 3541 ATTTGCCATGCTCCTTGCCGTTGACATCTTCTCAAATTTGAATGCCTTACCGGTACCG 3600
 TAAACGGTACGAGGAACGGCAACTGTAGAAGGAGTTTAACTTACGAGATGGCCATGGC
 f Y K G H E K G N V D E E F N F A E V P V -
 3601 TCGCGGCACCGTAAGGCAGTCCGCCAAGCAGTGGGGCAGGGCCAGGGCGATATAGCGGG 3660
 AGCGCCGTGGCATTCCGTGACGCGGTTTCGTGACCCCGTCCCGGTCCCGCTATATCGCCC
 f T A A G Y P L R G L V H P L A L A I Y R -
 3361 CATCCTCCGACTGGCGCAGCGATTTCCACTTGGCATACTCAACCGTATCAAAGCCTTTGG 3720
 GTAGGAGGCTGACCGCGTCGCTAAAGGTGAACCGTATGAGTTGGCATAGTTTCGGAAACC
 f A D E S Q R L S K W K A Y E V T D F G K -
 3721 CCAGATCCCTGGGGATCGACAGCTCTGTGAGGCTATCCAGTCCGAACATTCTTGCCTGG 3780
 GGTCTAGGGACCCCTAGCTGTGAGACAGTCCGATAGGTGAGGCTTGTAAAGGACGCGACC
 f A L D R P I S L E T L S D L G F M G A S -
 3781 CGGCAGAGAGGAAGGGAGCATGGGCTGCCGCCGCAACGTGTGAGATCTCCTCCAGCAGAA 3840
 GCCGTCTCTCCTTCCCTCGTACCCGACGGCGGGCTTGACACTCTAGAGGAGGTCTGTCTT
 f A A S L F P A H A A A A V H S I E E L L -
 3841 ACAGATCCTCCGGGTGGTTGCTGAAGTCAATCCCCGATCATGGCTGCAAAGGGGGCAC 3900
 TGTCTAGGAGGCCCCACCAACGACTTGAGTATTAGGGGCTAGTACCGACGTTTCCCCCGTG
 f F L D E P H N S F E Y D G I M A A F P A -
 3901 CACCAAAGGTGCCGTACTCCTCCTCATAGACTTTCTTGAACAGGGCGCTCTGATCAAAC 3960
 GTGGTTTCCACGGCATGAGGAGGAGTATCTGAAAGAACTTGTCCCGCAGACTAGTTTGA
 f G G F T G Y E E E Y V K K F L A S Q D F -
 3961 CGGCGGCACTCTTAAAGTCGCGGATCAGATCTTTTCGGCTGACATTGAGCATGCGGATTT 4020
 GCCGCCGTGAGAATTTGAGCGCCTAGTCTAGAAAAGCCGACTGTAAGTCGTACGCCTAAA
 f E A A S K F D R I L D K R S V N L M R I -

(Figure 3.7 con'd)

4021 TCAGGCCGGTACCGGTTTCGCTTTGATGTACCAGGTAATGCAGTCCACGCCATGACCCCTT 4080
 AGTCCGGCCATGGCCAAAGCGAACTACATGGTCCATTACGTACAGGTGCGGTACTGGGAA
 f K L G T G T E S Q H V L Y H L G R W S G -

4081 CCAGCTTTTGGAAAGCCGGCTCATGCATAATGGTGGACAGCTGGGCGGAGAGCAGCGCAT 4140
 GGTGAAAACCTTTCGGCCGAGTACGTATTACCACCTGTGACCCCGCCTCTCGTCGCGTA
 f E L K Q F A P E H M I T S L Q A S L L A -

4141 CGATCTGGGCAATACGTGCCTCAATACTGGCCAGTACGTACCGGAAACTGTCACCGTTC 4200
 GCTAGACCCGTTATGCACGGAGTTATGACCGGTATGCAGTGGCCTTTGACAGTGGCAAG
 f D I Q A I R A E I S A L V D G S V T V T -

4201 CCGACAGAACCTCACTTACCCATTCCCCAACAGGTCCCGCGCCCGCTCTCTCTCCAGGT 4260
 GGCTGTCTTGGAGTGAATGGGTAAGGGGGTTGTCCAGGGCGCGGCGAGAGAGAGTCCA
 f G S L V E S V W E G L L D R A R E R E L -

4261 CACTGCGTGCCATCCGGGTTTCAGCAATGATGCTATCCAGCAGGCTGTCCTCTGGAGCCG 4320
 GTGACGCACGGTAGGCCCAAAGTCGTTACTACGATAGGTTCGTCGACAGGAGACCTCGGC
 f D S R A M R T E A I I S D L L S D E P A -

4321 CGCTCTCGACAGCGGCACCTTTCTGGCAAGTTCTGTTGCTCATTATTGCTGTTCTCACT 4380
 GCGAGAGCTGTCCCGTGAAAGACCGTTCAGACAAGCGAGTAATAACGACAAGGAGTGA
 fd A S E V A A S E P L N Q E S M * Q Q E E S

4381 AAAACCGGTCTCTTTACCCAGATTTTGCAGTTTTTCGGTATCTTCAATGATGCCCTGCAA 4440
 TTTTGGCCAGAGAAATGGGTCTAAACGTCAAAAGCCATAGAAGTTACTACGGGACGTT
 d F G T E K G L N Q L K E T D E I I G Q L -

4441 TACCTCACCAGCGCTCGTTGCTGTACATCTTGTTTTTCAGATCGGAGAGTCGGCTGCG 4500
 ATGGAGTGGGTCCGCGAGCAACGACATGTAGAACAAAAGTCTAGCCTCTCAGCCGACGC
 d V E G L R E N S Y M K N K L D S L R S R -

4501 GATATTTCATCAGCTTGCCTAGTGGTTCGACCTGTGCGCTATTTCGCTCAGGAGTGAAATC 4560
 CTATAAGTAGTCGAACGCATCACCAGGTGACACGGCGATAAGCGAGTCCCTCACTTTAG
 d I N M L K R L P E V Q A A I R E P T F D -

4561 CGCCATACTTTGAAAATGCAGCTCCACCGACAGCTGGCTGTCATCTCCTGCCAGGGTATT 4620
 GCGGTATGAAACTTTTACGTGAGGTGGCTGTCGACCGACAGTAGAGGACGGTCCCATAA
 d A M S Q F H L E V S L Q S D D G A L T N -

4621 TTCAGTCCGGAATGACAGATGTGGATGTATCCCTTTGATAACATCGTTGAAGTTGTACAG 4680
 AAGTCACGCCTTACTGTCTACACCTACATAGGGAACTATTGTAGCAACTTCAACAGTGC
 d E T R F S L H P H I G K I V D N F N D R -

4681 ATCAATCGAAACGAATTTGCGTTCTTTACGCTTGGGAAGCGGGTTCGCCGTTGCCCTGA 4740
 TAGTTAGCTTTGCTTAAACGCAAGAAAGTCGAACCCCTTCGCCCCAGCGGCCAACGGGACT
 d D I S V F K R E K L K P L P T A P Q G S -

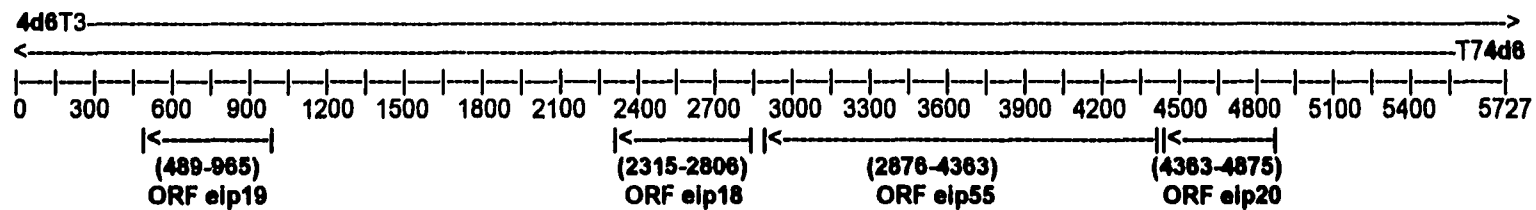
4741 ATAGTCACCGAGAACCCCATGACAAATGGCAGCTCCTTCATCTCCTGCGCATCGCCAAT 4800
 TATCAGTGGCTCTTGGGGTACTGTTTACCGTCGAGGAAGTAGAGGACGCTAGCGGTTA
 d Y D G L V G M V F P L E K M E Q A D G I -

4801 TTCAACATCATAGGTAATCTGCACTCTTGGCGAGCGGACCCGATCCAAAGTATGCTGCTT 4860
 AAGTTGTAGTATCCATTAGACGTGAGAACCGCTCGCCTGGGCTAGGTTTCATACGACGAA
 d E V D Y T I Q V R P S R V R D L T H Q K -

4861 GCTTTCACTTGCCATGAATTCATCTCCGTAACATTTCTTACAACACCGTATAATGTTATA 4920
 CGAAAGTGAACGGTACTTAAGTAGAGGCATTGTAAAGAATGTTGTGGCATATTACAATAT
 d S E S A M -

(Figure 3.7 con'd)

4921 GATGGGGATTATATGTGAATGAATTTGTCCATCTTTGTAAAAAATACATATTCATTTCAA 4980
 CTACCCCTAATATACACTTACTTAAACAGGTAGAAACATTTTTATGTATAAGTAAAGTT
 4981 TATCGACGATCCGATAATCCCCCGTATTCCACACTTTCCCTAGGGTAAAAACGGCGCAG 5040
 ATAGCTGCTAGGCTATTAGGGGGGCATAAGGTGTGAAAGGGATCCCATTTTTGCCGCGTC
 5041 ATGGCAGGCCGCTAATGTCTGGGTGAGGATGACTGTAACCGGGGACAGCCCCGAAAGGG 5100
 TACCGTCCGGCGATTACAGACCCACTCCTACTGACATTGGCCCCGTGTCGGGGGCTTTCCC
 5101 GGATTATTTTTGAAGTAATTAGTGCTATTTCAATGCTGAAAAGTTGTACCTACTAATATG 5160
 CCTAATAAAAACCTTCATTAATCACGATAAAGTTACGACTTTTCAACATGGATGATTATAC
 5161 CCAGACCGTCAGGTTCCGGAATGTAGAACTGTGTGGCTCCGCCAGGTAACATTGACCCCT 5220
 GGTCTGGCAGTCCAAGCCTTACATCTTGACACACCAGGGCGGTCCATTGTAACCTGGGGGA
 5221 ACTTTTTTTTTCCAAGGCGGCATTTCCGCGAGAGGAAAAATCGGCATCAATACGCGGCATT 5280
 TGAAAAAAAAGGTTCCGCCGTAAAGCCGCTCTCCTTTTTAGCCGTAGTTATGCGCCGTAA
 5281 CCTTTAAATCCACCGAACCAGGCATAGACGGGAACCTTTAGGGTTATTTCAAGCAACCAG 5340
 GGAAATTTAGGTGGCTTGGTCCGTATCTGCCCTTGGAATCCCAATAAAGTTCGTTGGTC
 5341 TCGAGAGAGTTCCAGTTTTCTTTGATAACGGAGGTGAGTCGTCCCCATTCCCTCATTGAT 5400
 AGCTCTCTCAAGGTCAAAAGAACTATTGCCTCCACTCAGCAGGGGTAAAGGAGTAACTA
 5401 ACTCCGTTTTTGCTTAGCTAATAGCATAAGCTGCTGCAGGCCTCCATCATGCCGGAAGGGC 5460
 TGAGGCAAAACGAATCGATTATCGTATGCGACGACGTCCGGAGGTAGTACGGCCTTCCCG
 5461 TGGATGGGACTCCACCAGGGAGACAGCAGGGTAGCATCAGTGACTTGGCGTCTTGACAAT 5520
 ACCTACCTGAGGTGGTCCCTCTGTCTGCCATCGTAGTCACTGAACCGCAGAACTGTTA
 5521 GCATTGCAACCATTGAATTTATATAATCCTGTCCCTGCAGGAAGAAGCTCTTTAGTTGGC 5580
 CGTAAGCTTGGTAACCTAAATATATTAGGACAGGGACGTCTTCTTCGAGAAATCAACCG
 5581 AAACCATACGATATTTTAAATGCATCGCAGACGGTGGTTCCTGGACCGGTAGAAATATTA 5640
 TTTGGTATGCTATAAAATTTACGTAGCGTCTGCCACCAAGGACCTGGCCATCTTTATAAT
 5641 CCGACATGGTTCCAGTCTAGATCAGAGTTCAAATAGACATAATGCGCTATCCTTTTTTCA 5700
 GGCTGTACCAAGGTCAGATCTAGTCTCAAGTTTTATCTGTATTACGCGATAGGAAAAAGT
 5701 GTAGATTTGAGCGTGCTATTATAGAAG 5727
 CATCTAAACTCGCACGATAATATCTTC



ORF elp19: putative 19 KD protein, possibly antigenic

ORF elp18: putative 18 KD protein, possibly antigenic

ORF elp55: putative 55 KD antigenic protein

ORF elp20: putative 20 KD protein, possibly antigenic

Figure 3.8: Schematic diagram of the insert of *E. ictaluri* genomic DNA in clone 4d6 double-strand sequenced starting from the T3 and T7 primers. Sequenced portions are denoted by arrows on top of the insert and putative ORF regions encoding antigenic proteins are denoted by arrows in the direction they are encoded.

Table 3.3: Analysis of possible open reading frames (ORF) encoded by the *Edwardsiella ictaluri* insert of clone 4d6. Estimated isoelectric points (pI) and molecular weights (MW) are shown with MW in kilodaltons. Partial ORFs and promoters are denoted with (p). Sequence locations of ORFs encoded in the reverse direction are denoted parenthetically. Putative ORFs encoding antigenic proteins are denoted with *.

Reading Frame	ORF	Location in Sequence	# of Amino Acids	MW	pI	Promoter
f	1(p)	(271-1)	90	10.3	9.92	no
f	2	(2185-974)	403	42.3	8.98	yes
f	3*	(2806-2315)	163	17.8	4.96	yes
f	4*	(4363-2876)	495	54.5	4.85	yes
e	5(p)	(485-1)	161	18.2	7.32	no
e	6*	(965-489)	159	18.8	4.78	yes
e	7	(5681-5169)	171	19.1	9.83	yes (p)
d	8*	(4875-4363)	171	19.5	5.09	yes
b	9	113-589	159	17.2	11.13	yes
b	10	2309-2692	128	13.4	9.33	yes
b	11	4103-4573	157	17.4	9.79	yes

Table 3.4: Comparison of the *Escherichia coli* σ^{70} promoter consensus sequence (Lewin 1990) to the putative promoter regions of the open reading frames (ORF) encoding putative antigenic proteins in the *E. ictaluri* insert of clone 4d6. The N designates an unspecified number of any bases.

Re-named (Original) ORF	-35	# bases between	-10	# bases between	+1 start site	# bases between	Shine-Deigarno
<i>E. coli</i>	TTGACA	16-19	TATAAT	5-9	A/G	N	A/G rich
eip55 (4)	TTGAAG	14	CAAAAT	7	A	16	GAGGAA
eip20 (8)	TTACCA	22	TATAAT	8	A	31	GGAGA
eip19 (6)	TCGACC	16	TAAAAA	5	A	6	GAG
eip18 (3)	ATCACA	18	TATCTT	5	A	2	AGAGG

Clone 1.4, 5d2 and 5d3 Sequence Data

The clones 1.4 and 5d2 contained the same inserts except that the insert in 1.4 was 207 bp larger at the T7 end, and the inserts were in opposite orientations behind the pBK-CMV promoter. The double-strand sequence data of the overlapping inserts of 5d2 and 5d3, and the T3 and T7 sequence data of the 1.4 insert are shown in Figure 3.9. A schematic diagram of the 1.4, 5d2 and 5d3 inserts is shown in Figure 3.10. The length of the entire region encoded by the three inserts was 6362 bp long after the vector sequence was eliminated, this sequence was designated 5d23.

The sequence data showed that the insert of 5d3 overlapped with the inserts of 1.4 and 5d2 by 2027 bp. The insert in 1.4, though not double-strand sequenced, was determined to be 4151 bp long by alignment of the T3 and T7 sequence data with the double-strand sequence of 5d2 and 5d3. The 1.4 insert encoded base pairs 1 to 4151 of the 6362 region. The insert in 5d2 was determined to be 3943 bp long and encoded base pairs 208 to 4152 of the 6362 region. The insert in 5d3 was determined to be 4238 bp long and encoded base pairs 2124 to 6362 bp of 6362 region (Figure 3.10).

Sequence comparison using BLAST showed a large region of homology with the *E. coli* genome in the 65 to 68 minute region (Accession U28377). The 6362 bp sequence of *E. ictaluri* had homology with a 15,443 bp region of *E. coli* which encoded the genes and ORF's *serA*, *rpiA*, *iciA*, *sbm*, *ygfD*, ORF_o275, ORF_o492, ORF_f303, *yggE*, *yggA*, *yggB*, *fda*, and *pgk*. Map identified nineteen potential ORFs in the six different reading frames of 5d23 sequence, many of which were overlapping. Comparison with Blast results showed that five complete ORFs and two partial ORFs of 5d23 had high identities with seven of the genes encoded by *E.coli*. The 5d23 sequence included partial sequences of the *serA* and *pgk* genes, and complete sequences of *rpiA*, *iciA*, *yggE*, *yggB* and *fda*. The regions encoding *E. coli* genes *sbm*, *ygfD*, ORF_o275, ORF_o492, ORF_f303 and *yggA* were not present in the 6362 bp 5d23 sequence. Gap was used to compare regions encoding the *E. coli* genes with the putative *E. ictaluri* genes, results are shown in Table 3.5.

```

1 TCTCCCAGCACCATCTCCGCCACCGAGCGGGTGTGGAAAACGGCGCGTTGAACACCGGG 60
AGAGGGTTCGTGGTAGAGGCGGTGGCTCGCCACAACCTTTTGCCGCGCAACTTGTGGCCC
d      G L V M E A V S R T N S F P A N F V P -

61 ACACCGCGGCGCTGCGCCGACGCCAGATCGACCTGATTGGTCCCGATACAAAAGCAGCCC 120
TGTGGCGCGCGACGCGGCTCGGTCTAGCTGGACTAACCAGGGCTATGTTTTTCGTGCGG
d      V G R R Q A A A L D V Q N T G I C F C G -

121 ACCGCCACCAGCTTTTCCGCCGCGGAAAACATCGCCTCACTCAGTTGGGTGCGCGAGCGC 180
TGGCGGTGGTTCGAAAAGCGGCGCCTTTTGTAGCGGAGTGAGTCAACCCACGCGCTCGCG
d      V A V L K E A A S F M A E S L Q T R S R -

181 AGACCGATAAAATGGGCATCGCGGATCGCCTCGCGCAGCGCATcGTCATCCAACGCTCCC 240
TCTGGCTATTTTACCCGTAGCGCCTAGCGGAGCGCGTCGCGTAgCAGTAGGTTGCGAGGG
d      L G I F H A D R I A E R L A D D D L A G -

241 TTATGGTATTCAATATTGTTATAACCGGCGGCGCAACGTATCGACGGCGCTTTGATGC 300
AATACCATAAGTTATAACAATATTGGCCGCGCGCGTTCGATAGCTGCCGCGAAACTACG
d      K H Y E I N N Y G A A R L T D V A S Q H -

301 ACCCCCTCCACCAGCAAAACTTAATTCTGTCTTGTCCAAAGAACTTTTGCCATGATC 360
TGGGGGAGGTGGTTCGTTTTGAATTAAGACAGGAACAGGTTTCTTTGAAAACGGTACTAG
d      V G E V L L F K I R D K D L S V K A M -

361 TGCCCTACTGTCCGAAAGGAAATGGTGTGCAATGTGCGGTTCTGTGCACCAGCTACGG 420
ACGGGATGACAGGCTTTCCTTTACCACACGTTTACACGCCAAGGACACGTGGTCGATGCC

421 CATTGAGCGTCCACCACTGCGCAACGCGCTTTATCAACATAACAAAAATTACACTCGCG 480
GTAACTCGCAGGTGGTGACGCGTTGCGGCGAAATAGTTGTATTGTTTTAATGTGAGCGC

481 ACAAGCGATGGGCGGGCGATAAACATCGTCACGCCGATAGCGCAATAGGCATAAACTGGT 541
TGTTTCGCTACCCGCCCGCTATTTGTAGCAGTGCGGCTATCGCGTTATCCGTATTTGACCA

541 AAAAAATATTAATAAGACGGAATGTATCAGCGGGAAAAATTAATCGGCTAAACTCTGTG 600
TTTTTTATAATTATTCTGCCTTACATAGTCGCCCTTTTTTAACCTAGCCGATTGAGACAC

601 ATAGATGTCACCGAATTTACACACAGCAGAAAAAATGTCCGCCGAGGCACAGCGCCCC 660
TATCTACAGTGGCTTAAAGTGGTGTCTGCTTTTTTTTACAGGCGGCGTCCGTGTGCGGGG

661 GCAACGGCGGGGACCCAGAGGCGATTATAGGGTCAGCGTTTTGACGCCATCCGCGCAGC 720
CGTTGCCGCCCGTGGGTCTCCGCTAATATCCAGTCGCAAACTGCGGTAGGCGCGTCTG
e      * L T L T K V G D A C -

721 CGACCAGCGCAACGTCCGCCACCGCGGTTGGCAAACAGCCCCACCGTCACGACGCCGGCGA 780
GCTGGTTCGCGTTGCAGCCGTGGCGCCAACCGTTTGTGCGGGTGGCAGTGCTGCGGCCGCT
e      G V L A V D A G R N A F L G V T V V G A -

781 TGGCGTTAATGGCGTCTCCAGTTTGCACGGCTCCATGATCTGCAGGTTGTGTACATCCA 840
ACCGCAATTACCGCAAGAGGTCAAACGTGCCGAGGTACTAGACGTCCAACACATGTAGGT
e      I A N I A N E L K C P E M I Q L N H V D -

841 GGATCAGTTGCCATTGTGCGGTGAGTACGCCCTGACGATACACCGGCTGCCCGCCCAGCT 900
CCTAGTGCAACGGTAACAGCCAGTCATGCGGGACTGCTATGTGGCCGACGGGCGGGTCGA
e      L I V N G N D T L V G Q R Y V P Q G G L -

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Figure 3.9: Double-strand sequence of *Edwardsiella ictaluri* overlapping inserts in clones 1.4, 5d2 and 5d3 with the deduced amino acid sequences of open reading frames encoding proteins homologous to *Escherichia coli* proteins, some of which are antigenic (d, e and f denote reading frames).

(Figure 3.9 con'd)

901 TCACCAGCTCACGGGCAACATAGGCGCGGCCATCGGGATCACCTCGACCGGTAGCGGGA 960
 AGTGGTTCGAGTGCCCGTTGTATCCGCGCGCGGTAGCCCTAGTGGAGCTGGCCATCGCCCT
 e K V L E R A V Y A R A M P I V E V P L P -
 961 AGCTACCCAGAATATCGACCTGTTTAGAGGCATCGACGATACAGACGAACCTTCTTGCCCA 1020
 TCGATGGGTCTTATAGCTGGACAAATCTCCGTAGCTGCTATGTCTGCTTGAAGAACCAGGT
 e F S G L I D V Q K S A D V I C V F K K A -
 1021 CCGCCGCCACGATCTTCTCACGTGTCAGCGCGCGGCCGCCGCCCTTGATCATCTGCATGG 1080
 GCGCGCGGTGCTAGAAGAGTGACAGTCCGCCCGCGCGCGGGAAGTGTAGACGTACC
 e V A A V I K E R T L A A G G G K I M Q M -
 1081 CGCCGTTGATCTCATCGGCCCCATCCACATAGATATCCAGCGAATCCACCTCATTGAGGT 1140
 GCGGCAACTAGAGTAGCCGGGTAGGTGTATCTATAGGTGCGTTAGGTGGAGTAACTCCA
 e A G N I E D A G D V Y I D L S D V E N L -
 1141 CAAAGACCGGGATCCCCAGGCTTTTCAGCCTGGCGGTGGAGGCATCAGAGCTGGAGACCG 1200
 GTTCTGGCCCTAGGGGTCCGAAAAGTCGGACCGCCACCTCCGTAGTCTCGACCTCTGGC
 e D F V P I G L S K L R A T S A D S S S V -
 1201 CACCCCTCGATCTGGCCCTTCATGGTCGCCAGCGCATCAATAAAGTGTGAGGCGGTGGAGC 1260
 GTGGGAGCTAGACCGGGAAGTACCAGCGGTGCGGTAGTTATTTACACTCCGCCACCTCG
 e A G E I Q G K M T A L A D I F H S A T S -
 1261 CGGTGCCTACCCCGACAATGGTCCCAGGGCGCACATACTTCAGCGCTGCCAGCCCACTG 1320
 GCCACGGATGGGGCTGTTACCAGGGTCCCGCGTGTATGAAGTCGCGACGGGTGCGGTGAC
 e G T G V G V I T G P R V Y K L A A W G V -
 1321 CTTTTTTCAATTCATCTGCGTCATACCGTATCCTATGGACTTTGCTACGAAAACGTGTG 1380
 GAAAAAAGTTAAGTAGGACGCAATGTCATAGGATACCTGAAACGATGCTTTTGCACAC
 e A K K L E D Q T M -
 1381 CGTTATTATAGAGCAGCGCGCATAAATACCCACAAACAGCGGCGTAAATTTACGCCTGCT 1440
 GCAATAATATCTCGTCGCGCGTATTTATGGGGTGTGTGCGCCGATTTAAATGCGGACGA
 1441 CTCTGTTTTTTTTCCCGTTTCACCCGCGTATTTTCTCCGCGACGCGCCGCAAAATGTGGCA 1500
 GAGACAAAAAAGGGCAAAGTGGGCGCATAAAAGAGGCGCTGCGGGCGGTTTTACACCGT
 1501 TATTGCGGTAGCGCCTCCAGCCGCCATGCGGAGCCTGAATAGGGCCCTCCGCTAGGGCG 1560
 ATAACGCCATCGCGGAGGTGCGCGGTACGCCTCGGACTTATCCCGGGAGGCGGATCCCGC
 1561 GACCATGCGCGCCGTATTCCCGACGTGAGCAATAACGTTTATACATAACAAAAATGGAAC 1620
 CTGGTACGCGCGGCATAAGGGCTGCACTCGTTATTGCAAATATGTATTGTTTTTACCTTG
 1621 CAAGGGGACAGATACCATAATGAAACGCCCGGACTACCGGACCTTGACGGCACTGGACGC 1680
 GTTCCCCTGTCTATGTTACTTTGCGGGCCTGATGGCCTGGAACGTCGCTGACCTGCG
 b M K R P D Y R T L Q A L D A -
 1681 CGTCATCCGCGAACGTGGTTTTGAGCGCGCTGCTCAAAAACCTTTGCATCACGCAGTCGGC 1740
 GCAGTAGGCGCTTGACCAAAAACCTCGCGGACGAGTTTTTGAACGTAGTGCCTGACCCG
 b V I R E R G F E R A A Q K L C I T Q S A -
 1741 GGTCTCCCAACGCATCAAGCAACTGGAACCTGTTTGGCCAGCCGCTGCTGGTACGTAC 1800
 CCAGAGGGTTGCGTAGTTGCTTGACCTTTTGGACAAACCGGTCGCGGACGACCATGCATG
 b V S Q R I K Q L E N L F G Q P L L V R T -
 1861 ACTGCTGGAGGAGGAGTGGCTGGGTAATGACAACAGCAGCGATGGTCCGCTGCTACTGTC 1920
 TGACGACCTCCTCCTCACCAGCCATTACTGTTGTGTCGCTACAGGCGACGATGACAG
 b L L E E E W L G N D N S S D G P L L L S -

(Figure 3.9 con'd)

1981 GGCCGACTCACCCTGCGCCTGAATCTGCAGGTGGAAGACGAAAGCCGACCCAGGAGCG 2040
 CCGGCTGAGTGGCGACGCGGACTTAGACGTCCAGCTTCTGCTTTTCGGCGTGGGTCTCTCGC
 b L A V N A D S L A T W L L P A L K P V L -

1921 GCTGGCAGTCAACGCCGACAGTCTGGCGACCTGGCTGCTGCCGGCCCTGAAGCCAGTGCT 1980
 CGACCGTCAGTTGCGGCTGTGACACCGCTGGACCGACGACGGCCGGGACTTCGGTCACGA
 b A D S P L R L N L Q V E D E S R T Q E R -

2041 CCTGCGCCGCGGTGAAGTGGTCGGCGCAGTGAGTCTGCAGCCCCAGCCGTGCCGAGCTG 2100
 GGACGCGGCGCCACTTCACCAGCCGCGTCACTCAGACGTCGGGGTCGGCGACGGCTCGAC
 b L R R G E V V G A V S L Q P Q P L P S C -

2101 CCTGGTGGATCGTCTGGGCGCGCTGGACTATCTGTTCTGCGCCTCCCCCGCCTTTGCCGC 2160
 GGACCACCTAGCAGACCCGCGGACCTGATAGACAAGACGCGGAGGGGGCGGAAACGGCG
 b L V D R L G A L D Y L F C A S P A F A A -

2161 CCGCTACTTCCCCAACGGGGTAACGCGCTCATCGTTACTGAAAGCGCCAGCGGTTCGCCTT 2220
 GCGGATGAAGGGGTTGCCCCATTGCGCGAGTAGCAATGACTTTCGCGGTTCGCCAGCGGAA
 b R Y F P N G V T R S S L L K A P A V A F -

2221 TGACCACCTGGACGACATGCATCAGGCTTTTCTACAGCAAACTTCGAAGTCCGCGCGGG 2280
 ACTGGTGGACCTGCTGTACGTAGTCCGAAAGGATGTCGTTTGAAGCTTGACGGCGGGCC
 b D H L D D M H Q A F L Q Q N F E L P P G -

2281 CAGCGTACCCTGTCATATCGTTAACTCATCCGAAGCGTTTGTACAGCTGGCGCTGCAGGG 2340
 GTCGCATGGGACAGTATAGCAATTGAGTAGGCTTCGCAACATGTCGACCGCGACGTCCC
 b S V P C H I V N S S E A F V Q L A L Q G -

2341 CACTACCTGCTGTATGCTGCCGCACCTGCAGGTTGAGCGCGAGCTGCGCAAAGGAGTACT 2400
 GTGATGGACGACATACGACGGCGTGGACGTCCAACCTCGCGCTCGACGCGTTTCTCATGA
 b T T C C M L P H L Q V E R E L R K G V L -

2401 GATCGATCTGACGCCGGGCATGGTCCAGCGCCGAATGCTGTACTGGCACCGTTTTGCCCC 2460
 CTAGCTAGACTGCGGCCCGTACCAGGTGCGGGCTTACGACATGACCGTGGCAAACGGGG
 b I D L T P G M V Q R R M L Y W H R F A P -

2461 GGAGAGCCGCCTGATGCGGCGGGTCACCGATGCGCTGATAAGCCACGGCCGCGACGTGCT 2520
 CCTCTCGGCGGACTACGCCGCCAGTGCGTACGCGACTATTGCGTGCCGGCGCTGCACGA
 b E S R L M R R V T D A L I S H G R D V L -

2521 GCGCCAGGACTAGACACTGGAGCTGCGCGCGGCAACGCGGGATCGGCAATCGATCCCGCC 2580
 CGCGGTCCTGATCTGTGACCTCGACGCGCGCGTTGCGCCCTAGCCGTTAGCTAGGGCGG
 b R Q D * -

2581 CACTATCGGCCCGTTATTTACTCAGGTTAAACACCACCTCAACCCGATCGTCGAAGTGAA 2640
 GTGATAGCCGGGCAATAAATGAGTCCAATTTGTGGTGGAGTTGGGCTAGCAGCTTCACTT
 e * K S L N F V V E V R D D F H -

2641 TCGTCTGCTGCTCATAGGTTTGTGCCGCCGAGGTTTGATTGGCGGACGGCGCCGCCGCGT 2700
 AGCAGACGACGAGTATCCAAACACGGCGGCTCCAAACTAACCGCCTGCCGCGGCGGCGCA
 e I T Q Q E Y T Q A A S T Q N A S P A A A -

2701 ACATGCGTGTCTCCGGCTCCGGCTGGAAGTTGGAGACGTGATAACGGACGCTATAGACCG 2760
 TGTACGCACAGAGCCGAGGCCGACCTTCAACCTCTGCACTATTGCCTGCGATATCTGGC
 e Y M R T E P E P Q F N S V H Y R V S Y V -

2761 GcCCCAACGTTACCCCAACCCCTGCGCCAGGGACTCCGCCTGGGATATCGCGTTCTCTGA 2820
 CgGGGTTGCAATGGGGTTTGGGGACGCGGTCCCTGAGGCGGACCCTATAGCGCAAGGACT
 e P G L T V G F G Q A L S E A Q S I A N R -

(Figure 3.9 con'd)

2821 TCGCCATCTGGCGCGCCTTATCGCGGTACACCTCAGGCTTGGCAACGCCAGCTCGACAG 2880
 AGCGGTAGACCGCGCGGAATAGCGCCATGTGGAGTCCGAACCGTTGCGGGTTCGAGCTGTC
 e I A M Q R A K D R Y V E P K A V G L E V -
 2881 AGCGGATCTCATTAAAGTTGGCCTTCAGCGCCCCGTCCAACAGACTGTTAAGCTTATCCA 2940
 TCGCCTAGAGTAATTCCAACCGGAAGTCGCGGGGAGGTTGTCTGACAATTGGAATAGGT
 e S R I E N L N A K L A G D L L S N L K D -
 2941 GCTGACGCAACGTCACCTGTACGGTACGCACGGCACGGTATCCCTTCAGCTGGCTTTTCC 3000
 CGACTGCGTTGCACTGGACATGCCATGCGTGCCGTGCCATAGGGAAGTCGACCGAAAAGG
 e L Q R L T V Q V T R V A R Y G K L Q S K -
 3001 CATCTTTCTGATACTCATACTCTGGCATGGTATACAGATTGGCGGCGCTGATATCCTGCG 3060
 GTAGAAAGACTATGAGTATGAGACCGTACCATATGTCTAACCGCCGCGACTATAGGACGC
 e G D K Q Y E Y E P M T Y L N A A S I D Q -
 3061 GGTGATGCGGTTACGCTTCAAAAAGTCAAAATATTTGGCTACCCGCTGATCGACCTGAG 3120
 CCAGCTACGGCAATGCGAAGTTTTTCAGTTTTTATAAACCGATGGGCGACTAGCTGGACTC
 e P D I G N R K L F D F Y K A V R Q D V Q -
 3121 ACTTGGCAACGGCGGCATCTTTAGCGCTGGCGTTAACCTGAAAGGTGAGGGTAGCCATGT 3180
 TGAACCGTTGCCGCCGTAGAAATCGCGACCGCAATTGGACTTTCCAGTCCCATCGGTACA
 e S K A V A A D K A S A N V Q F T L T A M -
 3181 CGGGAACGGCATCCACGCTGGCGGTACCCGATGTCACTACGTGCGGCCCCCTCTGGGACCT 3240
 GCCCTTGCCGTAGGTGCGACCGCCATGGGCTACAGTGATGCACGCCGGGAGACCCCTGGA
 e D P V A D V S A T G S T V V H P G E P V -
 3241 CCGCCGCTGAACACTGGCGGCCATGCCACCGACACCCATCATTGCAGCCAAGGCCAATA 3300
 GCGGGCGGACTTGTGACCGCCGGTACGGTGGCTGTGGGTAGTAACGTCGGTTCCGGTTAT
 e E A A Q V S A A M G G V G M M A A L A L -
 3301 CTGTTAATTTTCATGGTTATCCTCCGGTTCCTACTACGTGCGAGGGGCTTCCCTCTGT 3360
 GACAATTAAAGTACCAATAGGAGGCCAAGTGATGATGCAACGGTCCCCGAAGGGGAGACA
 e V T L K M -
 3361 CATGCGCCAGACCTAAGGTCCAACCCTATCCCGCGACCGATCGCCGACGAATGTGCTG 3420
 GTACGCGGTCTGGGATTCCAGGTTGGGATAGGGCGGCTGGCTAGCGGCTGCTTACACGAC
 3421 TTAGCATAGCGGCCTATGGCGCGACGTGAATCGGATATTCCCTACATAGCCGGTCATTG 3480
 AATCGTATCGCGCGGATACCGCGCTGCACCTAGCCTATAAGGGATGTATCGGCCAGTAAC
 3481 ACGCACCAACGATCGCGCAATAAACCACATAACTCCGCCGACAGCCATATTGATCGACCG 3540
 TCGGTGGTTGCTAGCGCGTTATTTGGTGTATTGAGGCGGCTGTCTGGTATAACTAGCTGGC
 3541 CTGGGCGCGCAAATGGCTCAGCCAGGCCACCTGCAGCGCCAGACCCTACCGAGGCTATTC 3600
 GACCCGCGCGTTTACCGAGTCGGTCCGGTGGACGTGCGGGTCTGGGATGGCTCCgATAAG
 3601 AGACCGCCCCAGATCACCAGCTTCAACGCGCGGCCGTGGAAGTCCCGGCGCAGATCAGC 3660
 TCTGGCGGGGCTAGTGGTGAAGTTGCGCGCCGCGCAGCTTCCAGGGCCGCTCTAGTCG
 3661 GCCGTACTCAGGATGAATCGCTGCACAAATGCAGAAAATATAACGATGCCCTTCAGAAG 3720
 CGGCATGAGTCTTACTTAGCGACGTGTTTACGTCTTTTATATTGCTACGGGGAAGTCTTC
 3721 AGGAGGAGCGGACGGTTAATAAAGAGAATCATAAACAGCCGGGCGAGCGGGGGAAATA 3780
 TCCTCCTCGCCTGCCAATTATTTCTCTTAGTATTGTGCGGCCGCTCGTGTCCCCCTTTAT
 3781 GCGATTTCTTATCGCAGATTAGACGGGCTCATGGTGTCTGGCCGGGGCGTCGGCCAGACG 3840
 CGCTAAAGAATAGCGTCTAATCTGCCCAGTACCACAGACCGGCCCCGAGCCGGTCTGC

(Figure 3.9 con'd)

3841 GGAGGAGATCACTCGGCACGGGCTTTGACAGCCTGATACAGATGCACGTCCATCTGTGGG 3900
 CCTCCTCTAGTGAGCCGTGCCCGAAACTGTCGGACTATGTCTACGTGCAGGTAGACACCC
 d * E A R A K V A Q Y L H V D M Q P -

3901 AACGGAATGCCAATATTATTGGCATCCAGCGCGCGTTTGAATTCTCCATCAGATCAAAA 3960
 TTGCCCTTACGGTTATAATAACCGTAGGTGCGCGCGCAAACCTTTAAGAGGTAGTCTAGTTTT
 d F P I G I N N A D L A R K F N E M L D F -

3961 TAGATGGCCCAATAGTCGGCGTTGTTGCCCCAGACACGCACCACAAAATTGAGCGACGAG 4020
 ATCTACCGGGTTATCAGCCGCAACAACGGGTCTGTGCGTGGTGTTTTAACTCGCTGCTC
 d Y I A W Y D A N N G W V R V V F N L S S

4021 GCCGCCATTTTCGTTCAGGCGAATGGTAACGCCATCATCGTGCAGAAATGCGCGTATCGGCC 4080
 CGGCGGTAAAGCAAGTCCGCTTACCATTGCGGTAGTAGCACGTCTTACGCGCATAGCCGG
 d A A M E N L R I T V G D D H L I R T D A -

4081 GCCACCACATCGCCAGCACCCGCTTGACCACGTCGATATCGGCGTCTGAGGCCACACCC 4140
 CGGTGGTGTAGCGGGTCTGGGCGAACTGGTGCAGCTATAGCCGAGCATCCGGTGTGGG
 d A V V D G L V R K V V D I D A D Y A V G -

4141 ACGATAATATCGATCCGGCGCTTCGGCTCGCGGGAGAAAGTTGATGATGTTGCCGGCAATG 4200
 TGCTATTATAGCTAGGCCGCGAAGCCGAGCGCCCTCTTCAACTACTACAACGGCCGTTAC
 d V I I D I R R K P E R S F N I I N G A I -

4201 ATCTTGCCATTGGGCACCACAATCACTTTATTGTGTCAGCTGTGGCCAGCGTGGTAGAGAAA 4260
 TAGAACGGTAACCCGTGGTGTAGTGAAATAACAGTCGACACCGGTCCACCACATCTCTTT
 d I K G N P V V I V K N D A T A L T T S F -

4261 ATCTGCACCTCGCGTACCGTGCCGGTGACGCCGCCAGATCCACGACCTCTCCCGCGCGG 4320
 TAGACGTGGAGCGCATGGCACGGCCACTGCGGCGGGTCTAGGTGCTGGAGAGGGCGCGCC
 d I Q V E R V T G T V G G L D V V E G A R -

4321 AACGGGCGGAACAGCACCAGCAGCAGCGCGCGGCAAAGTTGGACAACGACCCCTGTAGC 4380
 TTGCCCGCCTTGTGCTGGTCTGTCGCGCGCGCGCTTTCAACCTGTTGCTGGGGACATCG
 d F P R F L V L L V G A A F N S L S G Q L -

4381 GCCAGACCGACCGCCAAACCGGCGGCACCCAGCACAGCGATCACCGAGGTGGTCTGCACC 4440
 CGGTCTGGCTGGCGGTTTGGCCGCGTGGGTCTGTCGCTAGTGCTCCACCAGACGTGG
 d A L G V A L G A A G L V A I V S T T Q V -

4441 CCAAGCCGCCCCAGCGCGGCGATAATGGTAAAGGCCAGAATGGCATAACGTACCATCGCC 4500
 GGTTCGGCGGGGTCGCGCCGCTATTACCATTTCGGTCTTACCGTATTGCATGGTAGCGG
 d G L R G L A A I I T F A L I A Y R V M A -

4501 GCCAGGAAATGCACTACCGTCACATCCAGACCACGCGCCTTCAGCAGGCGAATCAGCGTC 4560
 CGGTCTTTTACGTGATGGCAGTGTAGGTCTGGTGC GCGGAAGTCGTCCGCTTAGTCGCAG
 d A L F H V V T V D L G R A K L L R I L T -

4561 GTCGAAATGCCGCGGGCGATCAGGGAACCGATGATCAGGATCACTACCGCCGCCACCAGA 4620
 CAGCTTTACGGCGCCGCTAGTCCCTTGGCTACTAGTCTTAGTGATGGCGGCGGTGGTCT
 d T S I G R A I L S G I I L I V V A A V L -

4621 TTGACGGCATAGCCCAGCAGCAAATCCTGGTTACGGACGATCCAACCGCGGCGCTCTGT 4680
 AACTGCCGTATCGGGTCTGCTTTAGGACCAATGCCGTGCTAGGTTGGCGGCGCGAGACA
 d N V A Y G L L L D Q N R V I W G G A S Q -

4681 ATGCCGTCAACTACATCCAATTCTTTCATCTTATTTTTTACCCTCTGATTTTATTACCTG 4740
 TACGGCAGTTGATGTAGGTTAAGAAAGTAGAATAAAAAATGGGAGACTAAAGTAATGGAC
 d I G D V V D L E K M -

(Figure 3.9 con'd)

4741 ACTCCGTCACCGTAACCCCGATAACACGGGCAAAACAGGCCGAGATCGCAGCCCAATGA 4800
TGAGGCAGTGGCATTGGGGCTATTGTGCCCGTTTTGTCCGGCTCTAGCGTCGGGTTACT

4801 TTATAAGGGTAACCCAACCTACGGCGATGAACTCCACATTTTCATTAACGCTAACAATCTGA 4860
AATATTCCCATTTGGGTTGATGCCGCTACTTGAGGTGTAAAGTAATTGCGATTGTTAGACT

4861 AAATTAACCATATAACACAACGTATTGATATGGCCGCTCGCGATGATATTTCCCTTTC 4920
TTTAATTTTGGTATATTGTGTGCATAACTATAACGGCGAGCGCTACTATAAAGGGAAAG

4921 CCGGCGATCTCCCGGCATAAAAAACCCGCAACGCGGGGTTTCTCCAGCCGATTGACGA 4980
GGCCGCTAGAGGGCCGTATTTTTTTGGGGCGTTGCGCCCCAAAGAGGTCCGGCTAACTGCT

4981 CGATTACAGCACGTCGATACAGTTCAGCTCTTTGAACGCCTGCTCCAGACGCTCGATCAT 5040
GCTAATGTCTGTCAGCTATGTCAAGTCGAGAACTTGGCGACGAGGTCTGCGAGCTAGTA
f * L V D I C N L E K F A Q E L R E I M -

5041 GCCAGTCTGAGCGGCACGCAGCCATACGCGTGGATCGTAGTACTTCTTGTTCGGCTTGTC 5100
CGGTCAGACTCGCCGTGCGTCGGTATGCGCACCTAGCATCATGAAGAACAAGCCGAACAG
f G T Q A A R L W V R P D Y Y K K N P K D -

5101 GTCGCCCTCCGGATTACCCAGCTGGCCCTGCAGATAACCTTCGTTCTTCTTGTAGTACTT 5160
CAGCGGAAGGCCTAATGGGTCGACCGGGACGTCTATTGGAAGCAAGAAGACATCATGAA
f D G E P N G L Q G Q L Y G E N K K Y Y K -

5161 CAGCACACCTTCCCAGGTTGCCCACTGGGTATCGGTGTCGATGTTTCATCTTCACCACACC 5220
-----+-----+-----+-----+-----+-----+
f GTCGTGTGGAAGGGTCCAACGGGTGACCCATAGCCACAGCTACAAGTAGAAGTGGTGTGG
L V G E W T A W Q T D T D I N M K V V G -

5221 GTAGCTGACCGCTTCTTTGATCTCTGCGCGGTAGAGCCGGAGCCACCGTGGGAAGACAAA 5280
CATCGACTGGCGAAGAACTAGAGACGGCGCCATCTCGGCCTCGGTGGCACCTTCTGTTT
f Y S V A E K I E A A T S G S G G H F V F -

5281 GTTCAGGCTGTTATGCGGCAGGTTATGCTTCTTGGAGACATACTCCTGAGAGTTTTTTCAG 5340
CAAGTCCGACAATACGCCGTCCAATACGAAGAACCTCTGTATGAGGACTCTCAAAAAGTC
f N L S N H P L N H K K S V Y E Q S N K L -

5341 GATCACCGGCGTCAGCTGCACGTTACCCGGCTTGTTACACGCGGTGCACGTTGCCGAAGGA 5400
CTAGTGGCCGCGAGTCGACGTGCAATGGGCGCAACATGTGCGGCACGTCGAACGGCTTCCT
f I V P T L Q V N G P K Y V G H V N G F S -

5401 GGCAGCGATGGTGAAACGCGGGCTGATGGCGCTCAGCTTGGTGAACGCGTAGTCCACGTC 5460
CCGTCGCTACCACTTTGCGCCGACTACCGCGAGTCGAACCACTTGGCGATCAGGTGCAG
f A A I T F R P S I A S L K T F A Y D V D -

5461 CTCCGGCTGGGTGTACAGCGCGGAGTTGTCCAGGTGACTGTTGTCCACACCGTCTTCTTC 5520
GAGGCCGACCCACATGTGCGCCTCAACAGGTCCACTGACAACAGGTGTGGCAGAAGAAG
f E P Q T Y L A S N D L H S N D V G D E E -

5521 ACCACCGGTGCAACCCAGCTCCAGCTCCAGGGTCATACCGATCTTGGACATGCGGGCCAG 5580
TGGTGGCCACGTTGGGTCGAGGTGCGAGGTCCAGTATGGCTAGAACCTGTACGCCCGGTC
f G G T C G L E L E L T M G I K S M R A L -

5581 GTACTGAGAGCAGATTTCAATGTTCTCTTCCAGAGACTCTTCGGAAGATCGATCATGTG 5640
CATGACTCTCGTCTAAAGTTACAAGAGAAGGTCTCTGAGAAGCCTTTCTAGCTAGTACAC
f S S F L P K G T A A F H K E G A D L L G -

5701 GTCCAGCCACGGCAGCAGCTTCTTGGCGCAGTGGTTCGGTATGCAGGATCACCGGAACACC 5760
CAGGTGGTGGCGTCGTCGAAGAACCGGTCACCGAGCCATACGTCCTAGTGGCCTTGTGG
f D L W P L L K K A C H D T H L I V P V G -

(Figure 3.9 con'd)

5761 GTAGTATTTCGGCCATCTGATGCACGTTGGTGGCACCAGGCGATCGCGCCGAGGATGGCCCC 5820
 CATCATAAGCCGGTAGACTACGTGCACCACGCGTGGCCGCTAGCGCGGCTCCTACCGGGG
 f Y Y E A M Q H V H H A G A I A G L I A G -
 5821 CTGCTGGCCCTCCAGCTTCAGTCCTTTACCGGCGATGAAGGCAGCACCGCCGTTGGAGAA 5880
 GACGACCGGGAGGTGGAAGTCAGGAAATGGCCGCTACTTCCGTCGTGGCGGCAACCTCTT
 f Q Q G E L K L G K G A I F A A G G N S F -
 5881 CTGAACGATGATCGGCGCGCGTACCTTGGCTGCAGCCTCCATCACCGCGTTGATGGAGTC 5940
 GACTTGCTACTAGCCGCGCGCATGGAACCGACGTCGGAGGTAGTGGCGCAACTACCTCAG
 f Q V I I P A R V K A A A E M V A N I S D -
 5941 CGTACCGACGCAGTTAACGGCCGGCAGAGCGAATTTGTTCTCTTTTCGCAACCTGGAATAC 6000
 GCATGGCTGCGTCAATCGCCGGCCGTCTCGCTTAAACAAGAGAAAGCGTTGGACCTTATG
 f T G V C N V A P L A F K N E K A V Q F V -
 6001 TTCTTGAACGTCATCGCCAAAGATGACACCCGGTTTAAACAAAGTCAAAGATTTTAGACAT 6060
 AAGAACTTGCAAGTAGCGGTTTCTACTGTGGGCCAAATTGTTTCAGTTTCTAAATCTGTT
 f E Q V D D G F I V G P K V F D F I K S M -
 6061 GTCTAATCGTCCTGTTTCGTGGGCGTGAGTGTTCCTCAATCGGATCAACATTACCGCGCC 6120
 CAGATTAGCAGGACAAAGCACCCGCACTCACAAAGGGTTAGCCTAGTTGTAATGGCGCGG
 6121 aAGGCGCGATAAAACaACgGGAgGcaTGATgGCCTCCCGTATCGGGCATTActGCTTGGC 6180
 tTCCGCGCTATTTTGtTGcCCTcCgtACTAcCGGAGGGCATAGCCCCTAATgaCGAACCG
 f * Q K A -
 6181 GCGCTCTTCGAGCATCACACAGCCGGCAGTTTCTTGCCCTTCCACAAACTCCAGGAAGGC 6240
 CGCGAGAAGCTCGTAGTGGTGTGCGCCGTCAAAGAACGGAAGGTGTTTGAGGTCCTTCCG
 f R E E L M V V A P L K K G E V F E L F A -
 6241 ACCGCGCGCGGTGGAGATGTAGGAGATCTTGTGCGCGATGCCGAACAGATCGATGGCCGC 6300
 TGGCGGCGGCCACCTCTACATCCTCTAGAACAGCCGCTACGGCTTGTCTAGCTACCGGCG
 f G G G T S I Y S I K D A I G F L D I A A -
 6301 CAGGGTATCGCCGCCCGGCGGATGGAGAAGGCGTCGCTGTTGGCGATAGCATTGGCCAC 6360
 GTCCCATAGCGGCGGCGGCGCTACCTCTTCCGAGCGACAACCGCTATCGTAACCGGTG
 f L T D G G G A I S F A D S N A I A N A V -
 6361 GA 6362
 CT
 f -

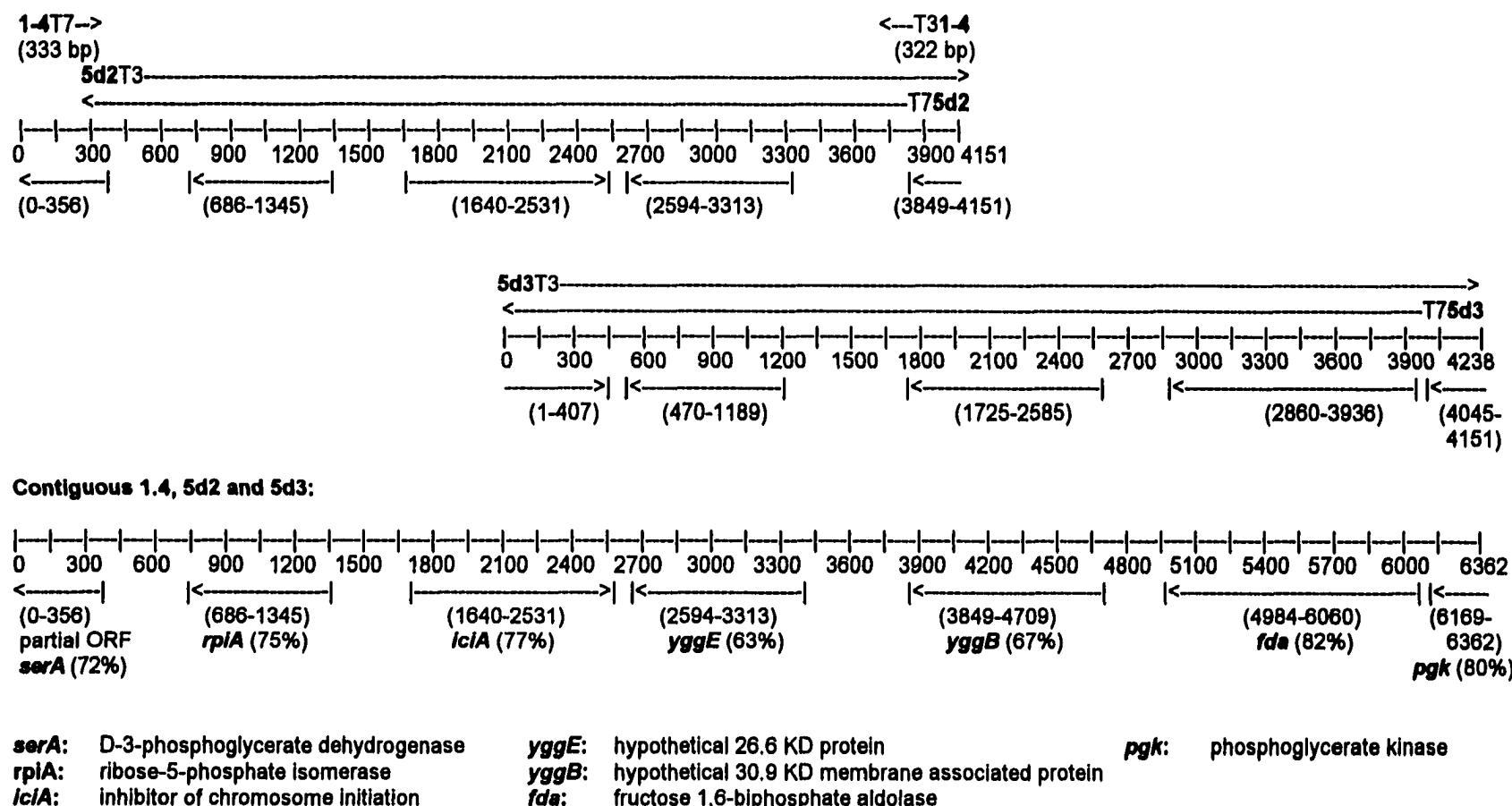


Figure 3.10: Schematic diagram of the overlapping inserts and contiguous sequence of *E. ictaluri* genomic DNA in clones 1.4, 5d2 and 5d3 sequenced from the T3 and T7 primers. The double-strand sequence starting from the T3 and T7 primers was obtained from the 5d2 and 5d3 inserts. Sequenced portions are denoted by arrows on top of the insert and the open reading frames of putative gene regions based on homologous sequence data with *Escherichia coli* is denoted by arrows in the direction they are encoded with the gene names and percent identity under the inserts.

The protein encoded by *serA* is D-3-phosphoglycerate dehydrogenase (PDGH; Accession P08328). This enzyme is a homotetramer responsible for the first committed step in the phosphorylated pathway of L-serine biosyntheses (Tobey and Grant 1986). The protein encoded by *rpiA* is ribose 5-phosphate isomerase A (RPIA; Accession P27252). This enzyme is a homodimer and part of the nonoxidative branch of the pentose phosphate pathway. The RPIA catalyzes the reversible conversion of D-ribose 5-phosphate to D-ribulose 5-phosphate (Hove-Jensen and Maigaard 1993). The protein encoded by *iciA* is ICIA (Accession P24194), which is a specific inhibitor of chromosomal initiation of replication *in vitro*. The ICIA is a homodimer that binds to three 13-mers in the origin(*oriC*) of *E. coli* to block initiation of replication (Thony, et al. 1991). The proteins encoded by *yggE* and *yggB* are hypothetical integral membrane proteins, with YGGE (Accession P1168) being 26.6 KD and YGGB being 30.9 KD (Accession P11666). The *fda* gene encodes fructose 1,6-bisphosphate aldolase (aldolase, Accession P11604). This enzyme catalyzes the sixth step in the glycolysis pathway which is the reversible conversion of D-fructose 1,6 bisphosphate to dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate (Stryer 1988; Alefounder, et al. 1989). The gene *pgk* encodes phosphoglycerate kinase (PGK, Accession P11665). This enzyme is a homodimer which catalyzes the second step in the second phase of the glycolysis pathway. Phosphoglycerate kinase catalyzes the reversible conversion of 1,3-bisphosphoglycerate and ADP into 3-phosphoglycerate and ATP (Stryer 1988).

The BLAST results also showed *E. ictaluri* encoded *fda* was also homologous with aldolase genes of a number of other bacteria and yeast including *Haemophilus influenza* (Accession U32734; Fleischmann, et al. 1995), *Euglena gracilis* (Accession X89769), *Neurospora crassa* (Accession L42380), *Schizosaccharomyces pombe* (Accession D17415; Mutoh and Hayashi 1994) and *Saccharomyces cerevisiae* (Accession X15003; Schwelberger, et al. 1989). The *E. ictaluri* encoded *iciA* was also homologous with the *iciA* gene of *Aeromonas salmonicida* (Accession ASU65741). The sequence identities are shown below in Table 3.6.

Putative promoter regions, identified manually, could be found for seven of the eight ORFs in the *E. ictaluri* 5d23 sequence. Putative transcriptional promoter regions of the seven

Table 3.5: Sequence gaps and nucleic acid (NA) and amino acid (AA) percent sequence identity and similarity between *Escherichia coli* genes and their *E. ictaluri* homologs encoded by the 5d23 sequence.

Gene	% NA Identity	Gaps	% AA Identity	% AA Similarity	Gaps
<i>serA</i>	71.9	0	78.6	88.0	0
<i>rpiA</i>	75.0	0	83.6	88.6	0
<i>iciA</i>	76.8	0	85.8	91.2	0
<i>yggE</i>	63.2	4	63.0	76.0	3
<i>yggB</i>	66.8	0	64.0	66.8	0
<i>fda</i>	82.1	1	87.7	91.3	1
<i>pgk</i>	80.0	0	92.1	93.7	0

Table 3.6: Sequence gaps and nucleic acid (NA) percent sequence identity between *fda* and *iciA* of other prokaryotes and yeast and their *E. ictaluri* homologs encoded by the 5d23 sequence.

Gene	Organism	% NA Identity	Gaps
<i>fda</i>	<i>Escherichia coli</i>	82.1	0
	<i>Haemophilus influenza</i>	63.0	1
	<i>Euglena gracilis</i>	59.9	3
	<i>Schizosaccharomyces pombe</i>	59.2	1
	<i>Saccharomyces cerevisiae</i>	56.1	3
	<i>Neurospora crassa</i>	40.6	7
<i>iciA</i>	<i>E. coli</i>	76.8	0
	<i>Aeromonas salmonicida</i>	61.0	1

ORFs are shown in Table 3.7 compared to the *E. coli* σ^{70} promoter consensus sequence (Lewin 1990). The promoter region for the *pgk* gene homolog, as well as most of the 5' end of the gene were upstream of the start of the *E. ictaluri* insert. A promoter region could be identified for *serA*, but most of the 3' end of the gene was downstream of where the *E. ictaluri* insert ended.

PC/Gene was used to determine molecular weight and pI of the putative protein encoded by each putative *E. ictaluri* gene. These results were compared to estimated molecular weights

Table 3.7: Comparison of the *Escherichia coli* σ^{70} promoter consensus sequence (Lewin 1990) to the putative promoter regions of cloned *Edwardsiella ictaluri* ORFs homologous to *E. coli* genes *serA*, *rpiA*, *iciA*, *yggE*, *yggA*, *fda*, and *pgk* in the 5d23 sequence. Regions that were not present are denoted by n.p. The N designates an unspecified number of any bases.

ORF	-35	# bases between	-10	# bases between	+1 start site	# bases between	Shine-Delgarno
<i>E. coli</i>	TTGACA	16-19	TATAAT	5-9	A/G	N	A/G rich
<i>serA</i>	TTGTCG	16	TATGTT	7	G	83	AGGG
<i>rpiA</i>	TTGTGG	20	TATAAT	7	A	21	AGGA
<i>iciA</i>	GTGAGC	18	AAAAAT	7	A	6	AGA
<i>yggE</i>	TTCACG	17	TATGCT	6	A	90	GGAGG
<i>yggB</i>	TTCATC	19	TATAAT	7	G	72	AAAAAA
<i>fda</i>	TTGTTT	18	TAATGT	7	G	27	AGGA
<i>pgk</i>	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.

reported for proteins encoded by *E. coli* genes obtained from the Swiss Protein data base. The pI values of proteins in the data base were determined using the Swiss 2D-Service. Theoretical pI values were computed and reported for ICIA, YGGE, and YGGB. Both the computed theoretical pI values, and actual values determined experimentally were reported for RPIA and aldolase. Comparisons of gene sizes, deduced protein sizes and estimated pI values are shown in Table 3.8. The actual values obtained for RPIA and aldolase are reported below.

The DNA sequence data of individual clones showed inserts of both 1.4 and 5.2 encoded partial ORFs of *serA* and *yggB* and complete ORFs of *rpiA*, *iciA* and *yggE*. The 5d3 insert encoded partial ORFs of *iciA* and *pgk*, and complete ORFs of *yggE*, *yggB* and *fda* (Figure 3.10). The partial ORF of *serA* in the pBK-ei1.4 and 5d2 inserts encoded a partial protein that was considered to be too small (6.4 KD) for further consideration. The partial ORF of *yggB* in the 5d2 insert could not be transcribed since it was missing the promoter and 5' region of the gene. The partial *yggB* of the 1.4 insert was in frame with the pBK-CMV promoter, with the first stop codon encountered being the *yggB* stop codon. The fusion protein encoded by the 302 bp, partial *yggB* gene of the 1.4 insert was estimated to be 11.2 KD, with a pI value of 4.23. The partial ORF of

Table 3.8: Comparison of *Escherichia coli* genes with their *Edwardsiella ictaluri* homologs. The number of base pairs (B.P) and deduced amino acids (A. A.) are given, as well as estimated molecular weight (MW) in kilodaltons and isoelectric points (pI). Partial genes are denoted by (p) gene sizes.

Gene	<i>Edwardsiella ictaluri</i> ORFs				<i>Escherichia coli</i> genes			
	# B.P.	# A.A.	MW	pI	# B.P.	#A.A.	MW	pI
<i>serA</i>	356 (p)	118	12.8	7.25	851	409	44.0	ne
<i>rpiA</i>	659	219	22.8	5.20	659	219	22.9	5.20
<i>iciA</i>	892	296	33.3	7.69	893	297	33.5	6.40
<i>yggE</i>	719	239	26.0	7.65	740	246	26.6	6.10
<i>yggB</i>	860	286	30.6	9.04	860	286	30.9	7.90
<i>fda</i>	1079	358	39.2	5.35	1079	358	39.0	5.52
<i>pgk</i>	193 (p)	63	6.4	4.20	1193	387	41.0	5.08

iciA encoded by the 5d3 insert could not be transcribed since it was missing the promoter region and 5' region of the gene and it was out of frame with the pBK-CMV promoter. Proteins that each of the three inserts could theoretically express based on whether the genes could be transcribed are shown in Table 3.9. These results are considered together with protein expression results in the section below entitled "Putative Identification of Genes Encoding Antigenic *E. ictaluri* Proteins".

Table 3.9: Proteins that *E. ictaluri* clones 1.4, 5d2 and 5d3 could theoretically express based on whether the genes could be transcribed. Partial proteins are denoted with (p). Proteins that could not be expressed are denoted with ne.

Clone	RPIA	ICIA	YGGE	YGGB	Aldolase
1.4	22.8	33.3	26.0	11.2 (p)	ne
5d2	22.8	33.3	26.0	ne	ne
5d3	ne	ne	26.0	30.6	39.2

Expression of *E. ictaluri* Proteins by Nine Selected Clones Analyzed by SDS-PAGE, 2D-PAGE and Western Blotting

Cloned antigenic proteins were visualized in SDS-PAGE gels Coomassie stained for total proteins, and in SDS-PAGE western blots probed with GAI-XA and CCS (Figures 3.11 and 3.12). Control lanes in SDS-PAGE gels and blots were loaded with XL0LR pBK-CMV (pBK) WCL. Protein expression of clone WCLs that were not IPTG-induced was evaluated in SDS-PAGE

western blots probed with GAI-XA. Results showed no difference in antigenic protein expression between induced and non-induced clones, indicating the proteins were under control of their own promoters (not shown).

The WCLs of the clones were also analyzed in 2D-PAGE gels silver stained for total proteins, and in 2D-PAGE western blots probed with GAI-XA and CCS. Control 2D-PAGE gels of pBK WCL were silver stained and western blotted with GAI-XA, CCS (Figure 3.13) and NCS. Results of SDS and 2D-PAGE western blots showed the CCS was highly cross-reactive with *E. coli* proteins. Absorption with *E. ictaluri* LPS did not remove any discernable cross-reactivity. A control 2D-PAGE western blot of pBK WCL probed with NCS was negative.

Preliminary 2D-PAGE silver stained gels run with SDS-PAGE silver stain standards in the sample lane and 2D-PAGE standards in the IEF tube gels showed bands and spots of like molecular weights lined up with each other in the gel. The 2D-PAGE standards also showed the pH gradient of the IEF tube gels ranged from pH 4.5 to 6.6. The molecular weights of antigenic proteins expressed by each clone were determined using molecular weight standards in SDS-PAGE gels and in the sample lane of 2D-PAGE gels. The pI values given for each cloned protein are subjective approximations reported for comparative purposes only. The molecular weight results are summarized in Table 3.10. Results of SDS and 2D-PAGE analysis of each clone are reported below.

Expression of Cloned Antigenic Proteins by Clone 1.1

In total protein, Coomassie stained SDS-PAGE gels, two cloned proteins were seen as bands with estimated molecular weights of 23 and 18 KD (Figure 3.12A). Cloned antigenic protein bands were not detected in SDS-PAGE western blots probed with GAI-XA (Figure 3.12B). In SDS-PAGE western blots probed with CCS, cloned protein bands of 23 and 18 KD were detected, though the 18 KD band was not always recognized (Figure 3.12C).

In a total protein, silver stained 2D-PAGE gel of clone 1.1 WCL, the 18 KD protein was not seen. The 23 KD protein was visualized as an elongated black spot with an approximated pI of 5.9 (Figure 3.14A). The 23 KD protein spot was detected faintly in a 2D-PAGE western blot

probed with GAI-XA, and it was detected strongly in a 2D-PAGE western blot probed with CCS (Figures 3.14B and 3.14C). In the western blot probed with CCS, three spots with molecular weights of approximately 23 KD were strongly detected with approximated pI values ranging from 5.0 to 5.9 (Figure 3.14C). Two proteins were also visualized and faintly detected in control gels of pBK WCL (Figure 3.13A), and only the spot running at a pI of 5.9 was unique to the 1.1 WCL. Since preliminary 2D-PAGE gels run with 2D standards showed many of the proteins used as standards ran as elongated spots with long tails sometimes extending along the whole pH gradient, it is likely recognition of the other two spots in the 1.1 WCL was due to a tail of the pI 5.9 spot running through the other two proteins.

Expression of Cloned Antigenic Proteins by Clone 5d4

In total protein, Coomassie stained SDS-PAGE gels, one cloned protein band of with an estimated molecular weight of 18 KD could be visualized (Figure 3.12A). The cloned protein band was detected in SDS-PAGE western blots probed with GAI-XA (Figures 3.12B) and CCS (Figure 3.12C). In a total protein, silver stained 2D-PAGE gel of clone 5d4 WCL, the 18 KD protein was visualized as a slightly elongated black spot with an approximated pI of 5.5 (Figure 3.15A). The 18 KD cloned protein spot was detected in 2D-PAGE western blots probed with GAI-XA and CCS (Figures 3.15B and 3.15C).

Expression of Cloned Antigenic Proteins by Clones 5d1, 5d8 and 6d5

In total protein, Coomassie stained SDS-PAGE gels, a unique protein band with an estimated molecular weight of 38 KD could be visualized in WCLs of clones 5d1, 5d8 and 6d5 (Figure 3.12A). A diffuse, broad band with an estimated molecular weight of 12 KD was detected detected in SDS-PAGE western blots in all three clone WCLs probed with GAI-XA (Figure 3.12B). In SDS-PAGE western blots probed with CCS, the 38 KD cloned protein band was detected in all three clone WCLs (Figure 3.12C).

Since clones 5d1, 5d8 and 6d5 all contained identical inserts and expressed the same cloned protein bands in SDS-PAGE western blots, only clone 6d5 was analyzed by 2D-PAGE. In a total protein, silver stained 2D-PAGE gel of 6d5 WCL, the 38 KD cloned protein was

visualized as a black oval spot with an approximated pI of 6.0 (Figure 3.16A). The 38 KD cloned protein spot was not detected in a 2D-PAGE western blot probed with GAI-XA, and it was detected faintly in a 2D-PAGE western blot probed with CCS (Figure 3.16B).

Expression of Cloned Antigenic Proteins by Clone 4d6

In total protein, Coomassie stained SDS-PAGE gels, three unique protein bands with estimated molecular weights of 63, 20 and 18 KD could be visualized (Figure 3.11A). Multiple protein bands were detected in SDS-PAGE western blots probed with GAI-XA, with the 63, 20 and 18 KD bands recognized the most strongly (Figure 3.11B). In SDS-PAGE western blots probed with CCS, unique antigenic protein bands of 63, 20 and 18 KD were detected, though the faintly detected 63 and 20 KD bands were not always recognized (Figure 3.11C).

In a total protein, silver stained 2D-PAGE gel of clone 4d6 WCL, the 63, 20 and 18 KD unique cloned proteins could all be visualized (Figure 3.17A). The 63 KD protein was seen as a small, elongated, dark brown spot with an approximated pI of 5.5. The unique 63 KD protein was difficult to discern because it ran almost on top of a spot that was present in both the pBK and 4d6 WCLs. The 20 KD protein was visualized as two small, translucent yellow spots that ran as a vertical doublet with an approximated pI of near 5.5. The 18 KD protein was visualized as a large, translucent orange elongated band that appeared as two to three running together horizontally, with long tails extending across the pH range, as well as a center tail extending up vertically so the spot ran as a large inverted "T". The main part of the protein had an approximated pI ranging from 5.0 to 5.5.

The 63, 20 and 18 KD unique protein spots were all detected strongly in a 2D-PAGE western blot probed with GAI-XA, with the inverted "T" pattern of the 18 KD protein being apparent (Figure 3.17B). It is likely recognition of multiple bands in the SDS-PAGE western blot probed with GAI-XA is due to the vertical tail of the 18 KD protein running through multiple other proteins. All three cloned proteins were also detected in 2D-PAGE western blots probed with CCS (Figure 3.17C).

Expression of Cloned Antigenic Proteins by Clones 1.4 and 5d2

In total protein, Coomassie stained SDS-PAGE gels, two unique bands with estimated molecular weights of 35 and 33 KD could be visualized in WCLs of both 1.4 and 5d2 (Figure 3.11A). An additional protein band with an estimated molecular weight of 16 KD could only be visualize in the 5d2 WCL. The 35 and 33 KD bands were detected strongly in both WCLs and the 16 KD band was detected strongly in the 5d2 WCL in SDS-PAGE western blots probed with GAI-XA and CCS (Figures 3.11B and 3.11C).

In a total protein, silver stained 2D-PAGE gels of 1.4 and 5d2 WCLs, unique proteins of 35, 33 and 27 KD could be visualized (Figure 3.18A). The 27 KD protein was seen as large, black, elongated spot with an approximated pI of 5.2. The 35 KD protein was seen as an elongated black spot and the 33 KD protein was seen as an elongated, clear yellow spot. Both the 35 and 33 KD spots ran at the edge of the gel where the pH gradient was approximately 6.6. The proteins appeared to have not entered, or only partially had entered into the IEF tube gel. The 16 KD protein seen in SDS-PAGE gels of 5d2 WCL could not be detected in the 2D-PAGE gel. The locations of the 35 and 33 KD proteins and the absence of the 16 KD protein may indicate that their pI values are outside the range of the pH gradient used in the IEF tube gels. In 2D-PAGE western blots probed with GAI-XA, the 27 KD protein was not detected, but both the 35 and 33 KD protein spots were detected strongly (Figure 3.18B). All three proteins were detected faintly in 2D-PAGE western blots probed with CCS. (Figure 3.18C).

Expression of Cloned Antigenic Proteins by Clone 5d3

In total protein, Coomassie stained SDS-PAGE gels, three unique protein bands with estimated molecular weights of 45, 33 and 19 KD could be visualized in the WCL of clone 5d3 (Figure 3.11A). The 45 KD band was detected faintly and the 33 and 19 KD bands were detected strongly in SDS-PAGE western blots probed with GAI-XA (Figure 3.11B). All three proteins were detected in SDS-PAGE western blots probed with CCS with only the 19 KD band being detected strongly (Figure 3.11C).

In a total protein, silver stained 2D-PAGE gel of the 5d3 WCL, unique proteins of 45, 45 and 33 KD could be visualized (Figure 3.19A). One of the 45 KD cloned proteins was visualized as a black, round spot with an approximated pI of 5.9. The second 45 KD protein was seen as an elongated dark spot, and the 33 KD protein was seen as an elongated, clear yellow spot, with both proteins located at the edge of the gel where the pH gradient was approximately 6.6. The 19 KD protein seen in SDS-PAGE gels of 5d2 WCL could not be detected in the 2D-PAGE gel. In 2D-PAGE western blots probed with GAI-XA, both 45 KD proteins were detected faintly and the 33 KD protein was detected strongly (Figure 3.19B). All three proteins were detected in 2D-PAGE western blots probed with CCS. (Figure 3.19C). The two 45 KD proteins are likely the same protein with the spot on the edge of the gel being protein that did not migrate into the tube gel during the first dimension IEF.

SAG Analysis of Antigenic Proteins Expressed by Clones 1.4, 5d2 and 5d3

Sequencing data had shown the DNA inserts of clones 1.4 and 5d2 were the same but inverted, with the insert of 1.4 being 207 bp longer at the T7 end, which corresponded with the T3 end of the 5d2 insert. Additionally, inserts of both clones overlapped with 2027 bp of the T3 end of the insert of 5d3, which was oriented in the same direction as the 5d2 insert (Figure 3.11). Specific-clone absorbed goat anti-*E. ictaluri* (SAG) serum was used in an effort to determine the relationships between the proteins expressed by these three clones, since SDS and 2D-PAGE gel and western blot results indicated some of the proteins expressed by the clones were the same proteins (Figures 3.11, 3.18 and 3.19).

Identical western blots, with WCLs of clones 1-4, 5d2 and 5d3 loaded in the sample lanes, were probed with GAI-XL that was further absorbed with induced WCLs of each specific clone. Specific clone absorbed antisera were designated SAG(pBK), SAG(1.4), SAG(5d2) and SAG(5d3). Results are summarized in Table 3.11. In the blot probed with SAG(pBK) results were the same as in blots probed with GAI-XA, with two antigenic bands with approximate molecular weights of 35 and 33 KD identified in the lane loaded with 1.4, three antigenic bands of 35, 33 and 16 KD identified in the lane loaded with 5d2 WCL, and three antigenic bands of 45, 33 and 19 KD identified in the lane loaded with 5d3 WCL.

Table 3.10: Estimated molecular weights in kilodaltons (KD) and isoelectric points (pI) of antigenic *Edwardsiella ictaluri* proteins expressed by *Escherichia coli* XL0LR pBK-ei clones. Protein recognition by goat anti-*E. ictaluri* (GAI) and/or convalescent catfish anti-*E. ictaluri* serum (CCS) is indicated.

Clone	Band/Spot molecular weights (KD)	Spot pI	Recognition by GAI or CCS
1.1	23	5.9	both
	18	-	CCS only
4d6	63	5.5	both
	20	5.5	both
	18	5.5	both
5d4	18	5.0-5.5	both
1.4	35	≥6.6	both
	33	≥6.6	both
	27	5.2	CCS only
5d2	35	≥6.6	both
	33	≥6.6	both
	27	5.2	CCS only
	16	-	both
5d3	45	5.9; 6.6	both
	33	≥6.6	both
	19	-	both
5d1	38	6.0	CCS only
	12	-	GAI only
5d8	38	6.0	CCS only
	12	-	GAI only
6d5	38	6.0	CCS only
	12	-	GAI only

Table 3.11: Recognition of antigenic proteins expressed by clones 1.4, 5d2 and 5d3 in SDS-PAGE western blots probed with specific-clone absorbed goat anti-*E. ictaluri* (SAG) serum. Molecular weight units are kilodaltons (KD).

Clone	Protein (KD)	SAG(PBK)	SAG(1.4)	SAG(5d2)	SAG(5d3)
1.4	33	++	-	-	+
	35	++	-	-	+
5d2	33	++	-	-	+
	35	++	-	-	+
	16	++	++	+	++
5d3	45	++	++	++	-
	33	++	-	-	-
	19	++	++	++	+

In the SAG(1.4), specific antibodies against the 35 and 33 KD cloned proteins expressed by the 1.4 clone should have been absorbed out of the serum. In the blot probed with SAG(1.4), the 33 and 35 KD proteins were no longer detected in any of the clones. These results appeared to indicate the 35 KD protein was the same in both 1.4 and 5d2, and the 33 KD protein was the same for all three clones.

In the SAG(5d2), specific antibodies against the 35, 33 and 16 KD cloned proteins expressed by the 5d2 clone should have been absorbed out of the serum. In the blot probed with SAG(5d2) the 16 KD protein of clone 5d2 was still recognized, but at a slightly lower level of detection, indicating not all antibodies against this protein had not been removed. The 33 and 35 KD proteins were no longer detected in any of the clones.

In the SAG(5d3) specific antibodies against the 45, 33 and 19 KD cloned proteins expressed by clone 5d2 should have been absorbed out of the serum. In the blot probed with SAG(5d3), the 19 KD protein of clone 5d2 was still recognized, but at a slightly lower level of detection, indicating not all of the antibodies against this protein had been removed. The 45 and 33 KD proteins of the 5d3 WCL were no longer detected, and the detection level of both the 33 and 35 KD proteins in 1.4 and 5d2 WCLs appeared to be decreased. These results again

appeared to indicate the 35 KD protein was the same in both 1.4 and 5d2, and the 33 KD protein was the same for all three clones.

Comparisons of Cloned *E. ictaluri* Proteins with Proteins in an *E. ictaluri* WCL Using SAG

Specific-clone absorbed goat anti-*E. ictaluri* (SAG) serum was used in an effort to indirectly associate antigenic *E. ictaluri* proteins expressed by the nine clones with antigenic proteins expressed by *E. ictaluri* cells. Identical 2D-PAGE western blots of *E. ictaluri* WCL were probed with GAI-XL that was further specifically absorbed with induced WCLs of each of the nine clones with SAG(pBK) used as the control. There were no discernable differences between the *E. ictaluri* blot probed with SAG(pBK) and any of the other SAG with the exception of the blot probed with SAG(4d6).

In the SAG(4d6), specific antibodies against the 63, 20 and 18 KD cloned proteins expressed by the 4d6 clone were absorbed out of the serum (Figure 3.20). Results showed that three bands and four corresponding spots which were strongly recognized in the control blot probed with SAG(pBK) were not detected in the blot probed with the SAG(4d6). The bands and spots no longer detected were a 63 KD band and a corresponding 63 KD spot, a 20 KD band and a corresponding 20 KD spot and an 18 KD band and two corresponding 18 KD spots. The pI values of the spots were all around 5.5 and their locations in the 2D-PAGE gel corresponded directly with molecular weights, pI values and migration patterns of cloned antigenic proteins expressed by clone 4d6 (Figure 3.17B).

Protein Sequencing

In an effort to confirm if antigenic proteins expressed by clone 4d6 were the same as those expressed by *E. ictaluri* cells, the 63 and 18 KD spots of the *E. ictaluri* WCL were cut from 2D-PAGE immobilon blots and submitted for automated N-terminal amino acid sequencing to see if their amino acid sequences matched those translated from DNA sequences of the cloned proteins. The automated N-terminal amino acid sequencing of both the 63 and 18 KD *E. ictaluri* spots was unsuccessful. The failure was most likely due to N-terminal blockage of the proteins, since proteins blocked at the N-terminal cannot be sequenced directly.

Figure 3.11: Whole cell lysates of induced pBK and clones 4d6, 1.4, 5d2 and 5d3 separated by SDS-PAGE (A) Total proteins in Coomassie Blue stained gel, (B) Antigenic proteins in western blot probed with GAI-XA and (C) Antigenic proteins in western blot probed with CCS. Molecular weight reference points (in kilodaltons) are shown. Lines point to cloned antigenic *Edwardsiella ictaluri* proteins expressed by each clone.

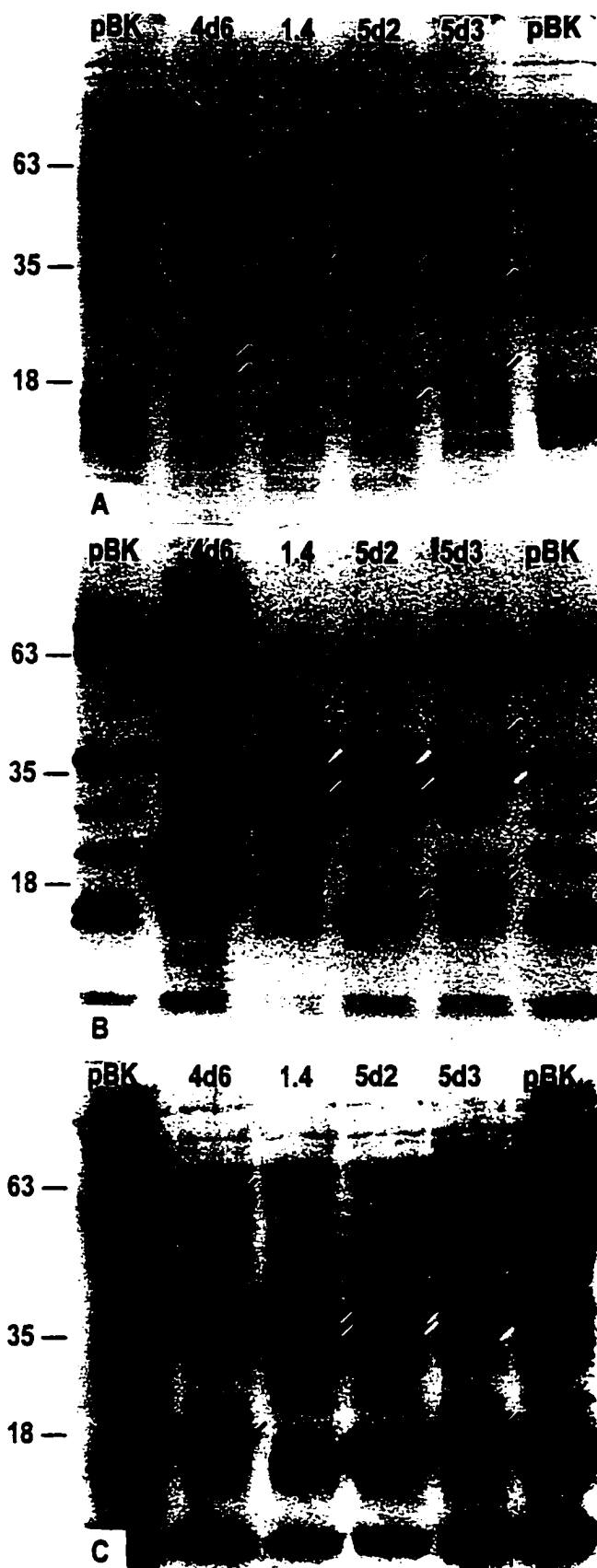


Figure 3.12: Whole cell lysates of induced pBK and clones 1.1, 5d1, 5d8 and 6d5 separated by SDS-PAGE (A) Total proteins in Coomassie Blue stained gel, (B) Antigenic proteins in western blot probed with GAI-XA and (C) Antigenic proteins in western blot probed with CCS. Molecular weight reference points (in kilodaltons) are shown. Lines point to cloned antigenic *Edwardsiella ictaluri* proteins expressed by each clone.

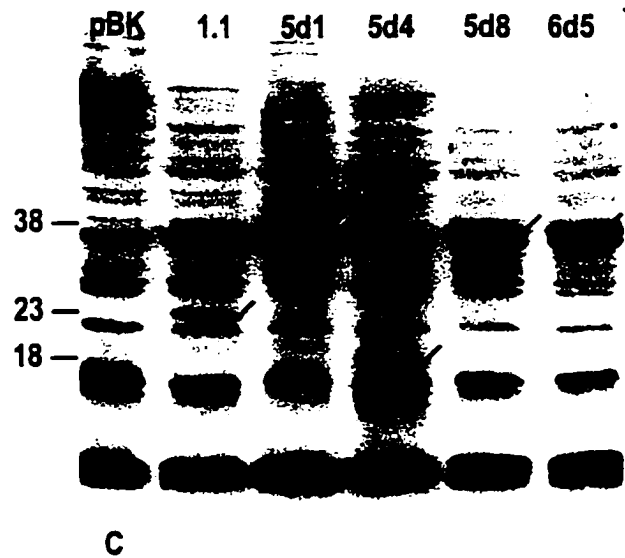
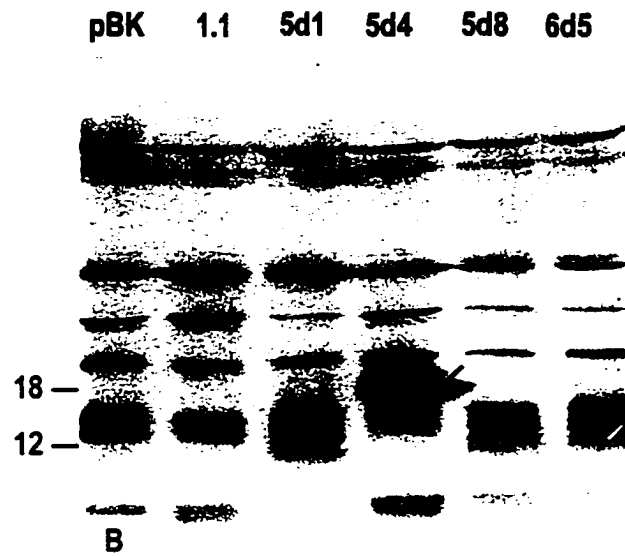
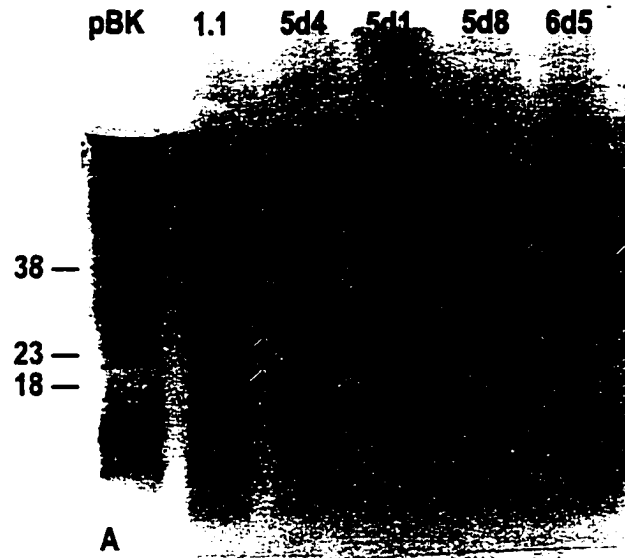


Figure 3.13: Whole cell lysate of induced pBK separated by 2D-PAGE (A) Total proteins in silver stained gel, (B) Antigenic proteins in western blot probed with GAI-XA and (C) Antigenic proteins in western blot probed with CCS.

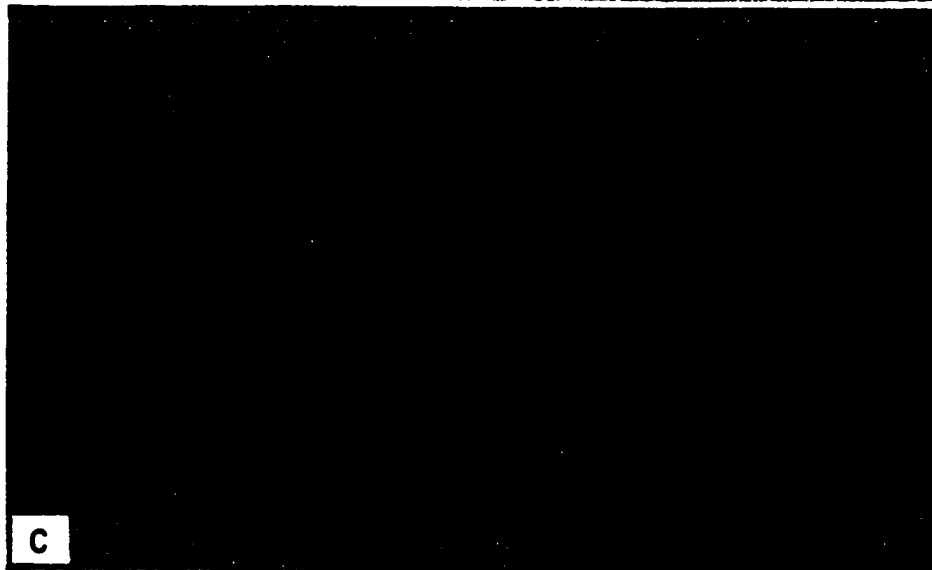
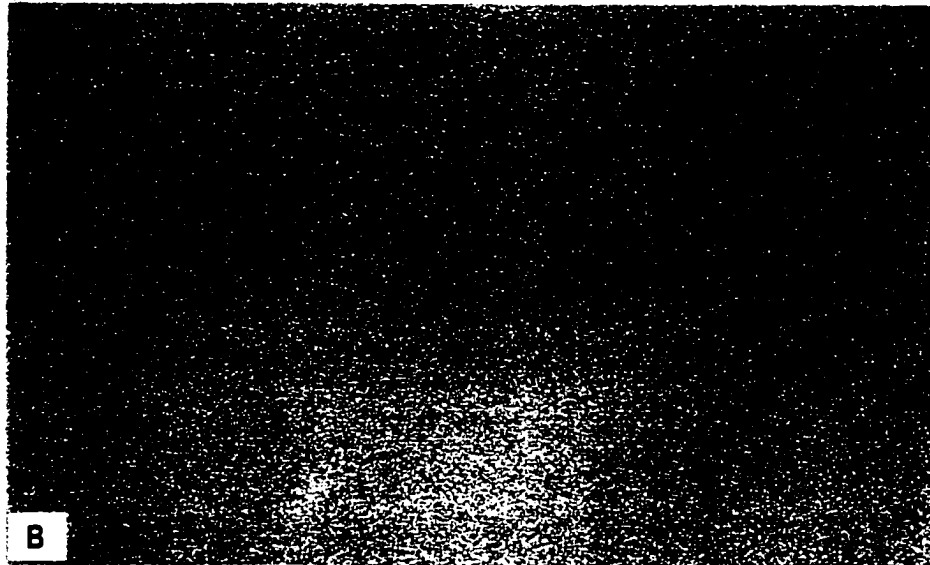


Figure 3.14: Whole cell lysate of induced clone 1.1 separated by 2D-PAGE (A) Total proteins in silver stained gel, (B) Antigenic proteins in western blot probed with GAI-XA and (C) Antigenic proteins in western blot probed with CCS. The 23 kilodalton cloned antigenic *Edwardsiella ictaluri* protein expressed by this clone is marked by its molecular weight.

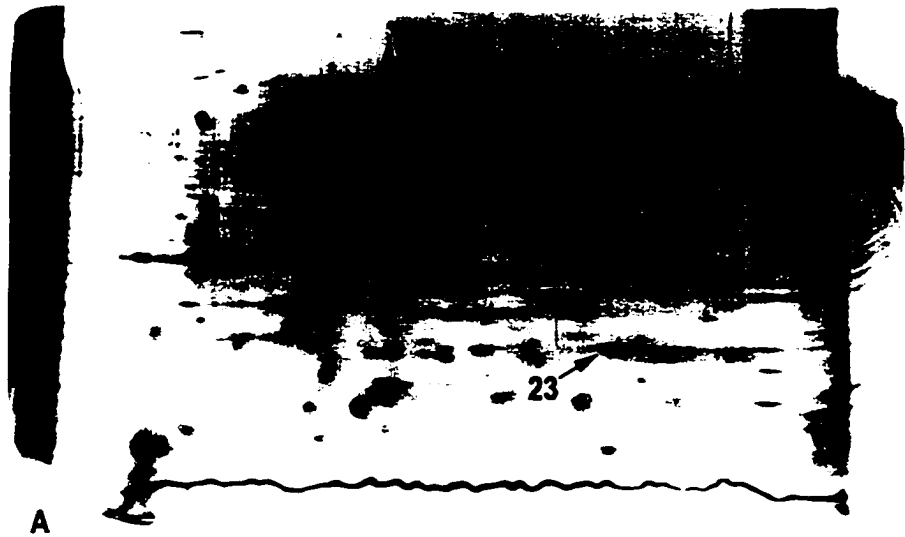


Figure 3.15: Whole cell lysate of induced clone 5d4 separated by 2D-PAGE (A) Total proteins in silver stained gel, (B) Antigenic proteins in western blot probed with GAI-XA and (C) Antigenic proteins in western blot probed with CCS. The 18 kilodalton cloned antigenic *Edwardsiella ictaluri* protein expressed by this clone is marked by its molecular weight.

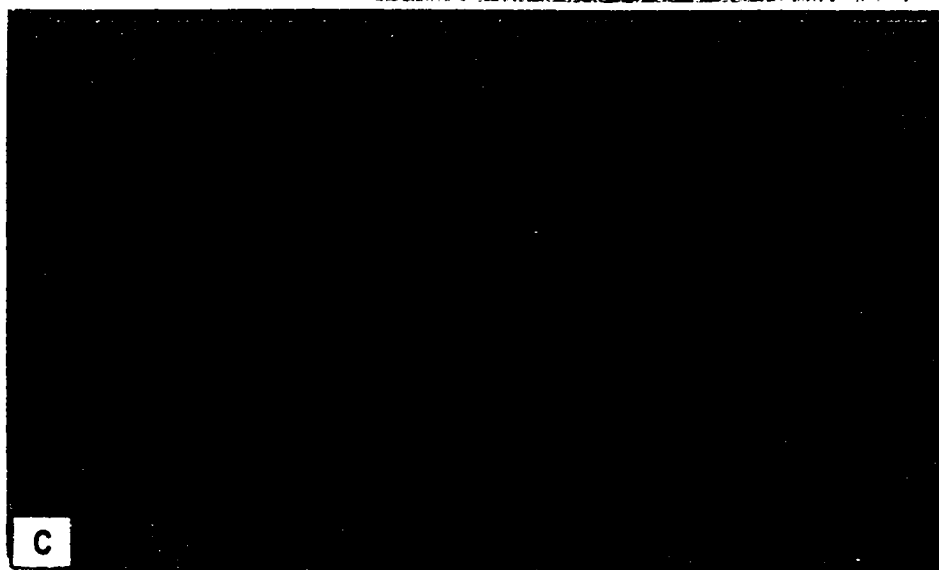
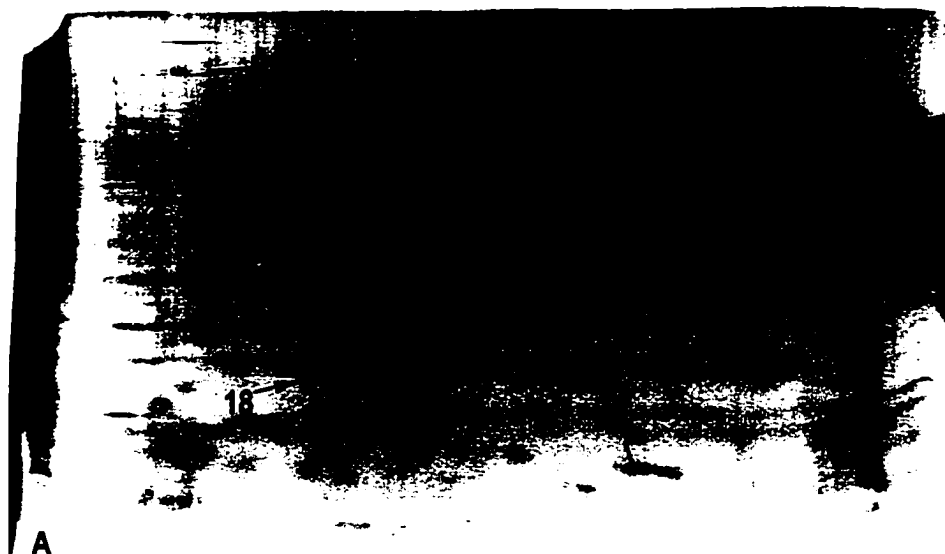


Figure 3.16: Whole cell lysate of induced clone 6d5 separated by 2D-PAGE (A) Total proteins in silver stained gel, (B) Antigenic proteins in western blot probed with CCS. The 38 kilodalton cloned antigenic *Edwardsiella ictaluri* protein expressed by this clone is marked by its molecular weight.

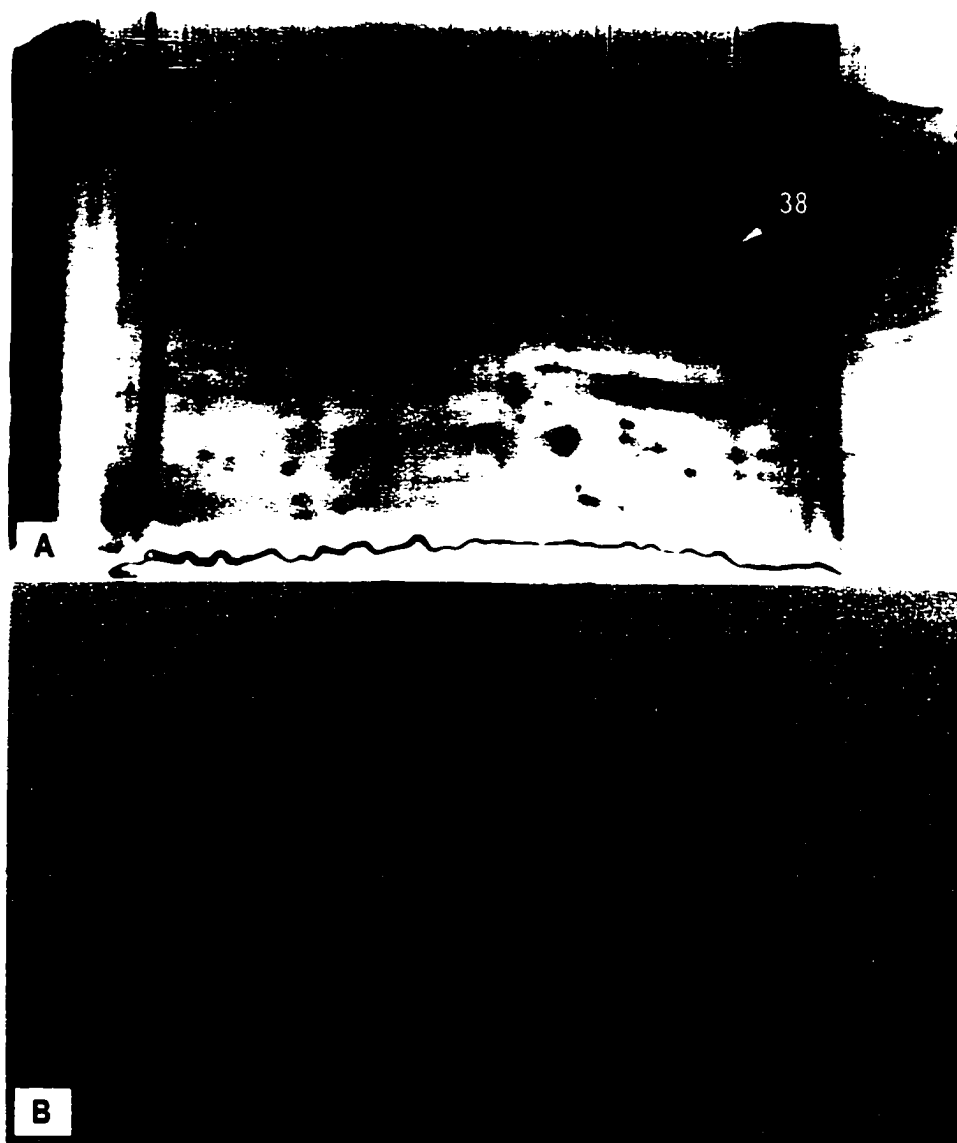


Figure 3.17: Whole cell lysate of induced clone 4d6 separated by 2D-PAGE (A) Total proteins in silver stained gel, (B) Antigenic proteins in western blot probed with GAI-XA and (C) Antigenic proteins in western blot probed with CCS. The 63, 20 and 18 kilodalton cloned antigenic *Edwardsiella ictaluri* proteins expressed by this clone are marked by their molecular weights.

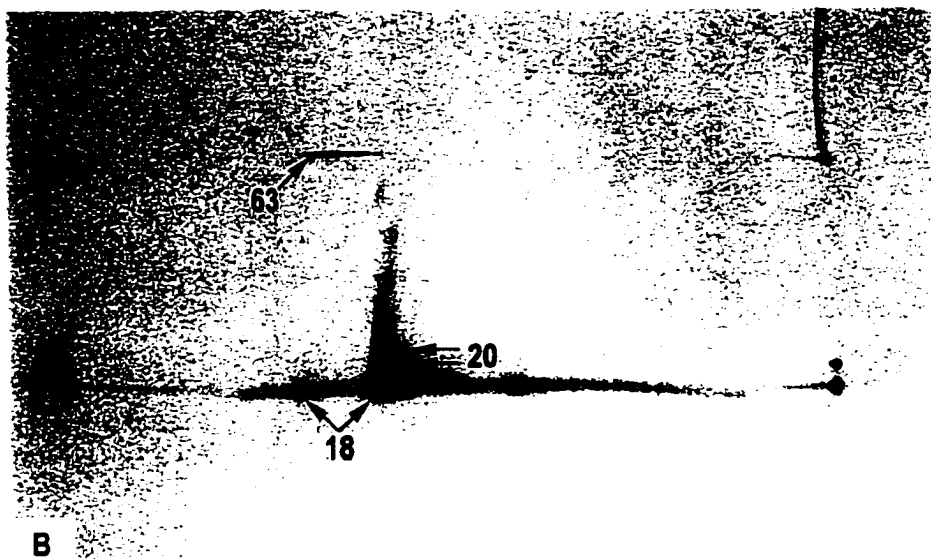


Figure 3.18: Whole cell lysate of induced clone 5d2 separated by 2D-PAGE (A) Total proteins in silver stained gel, (B) Antigenic proteins in western blot probed with GAI-XA and (C) Antigenic proteins in western blot probed with CCS. The 27, 33 and 35 kilodalton cloned antigenic *Edwardsiella ictaluri* proteins expressed by this clone are marked by their molecular weights.

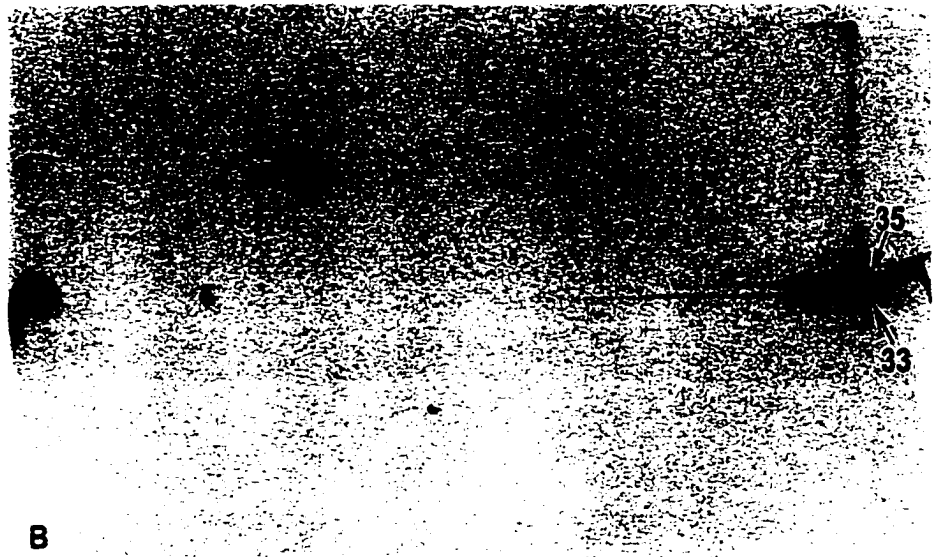
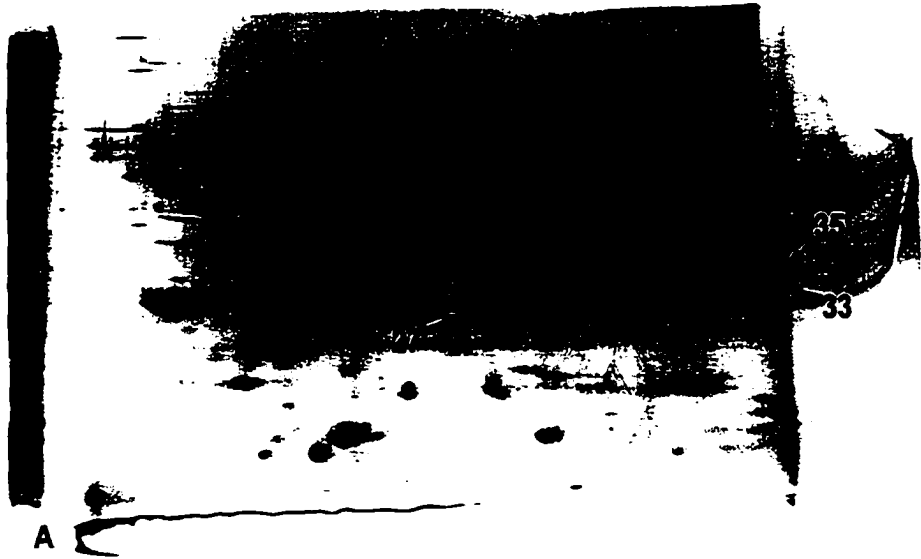
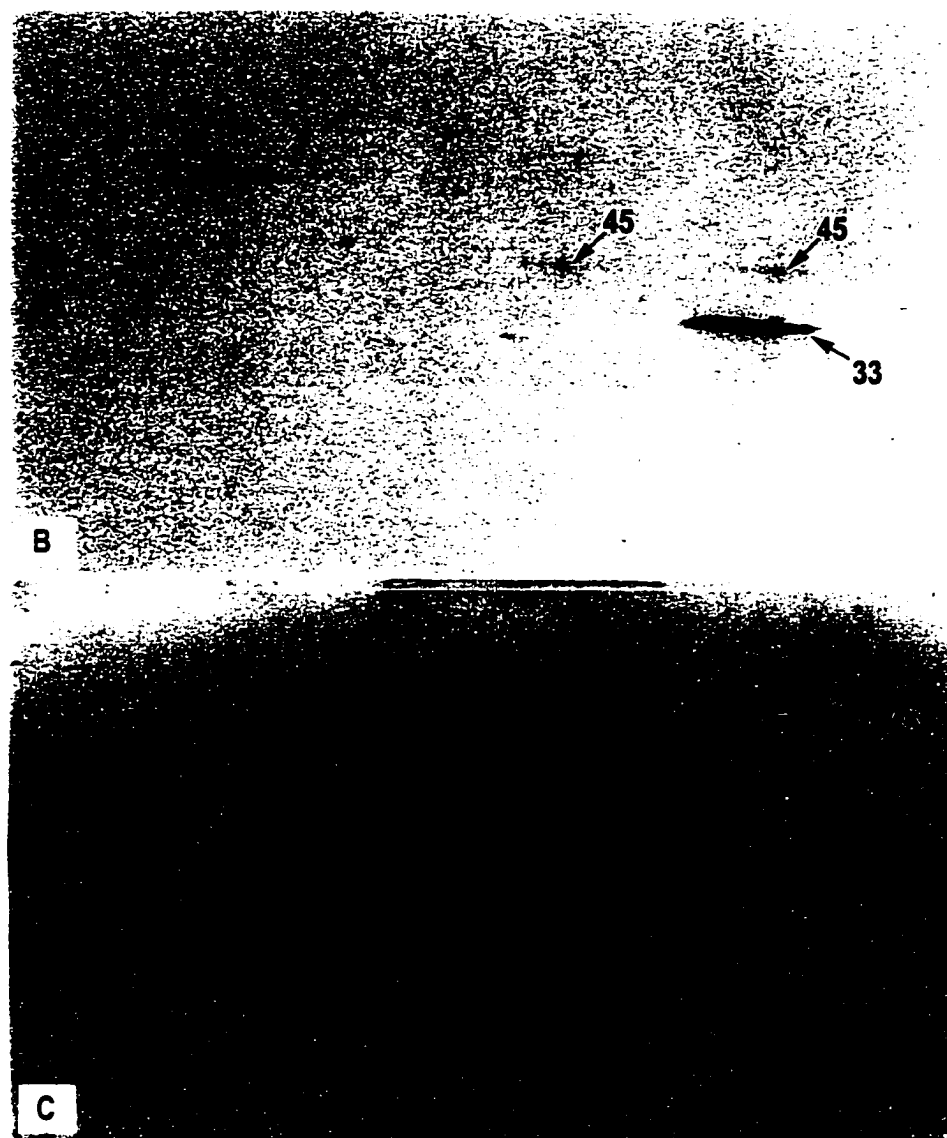
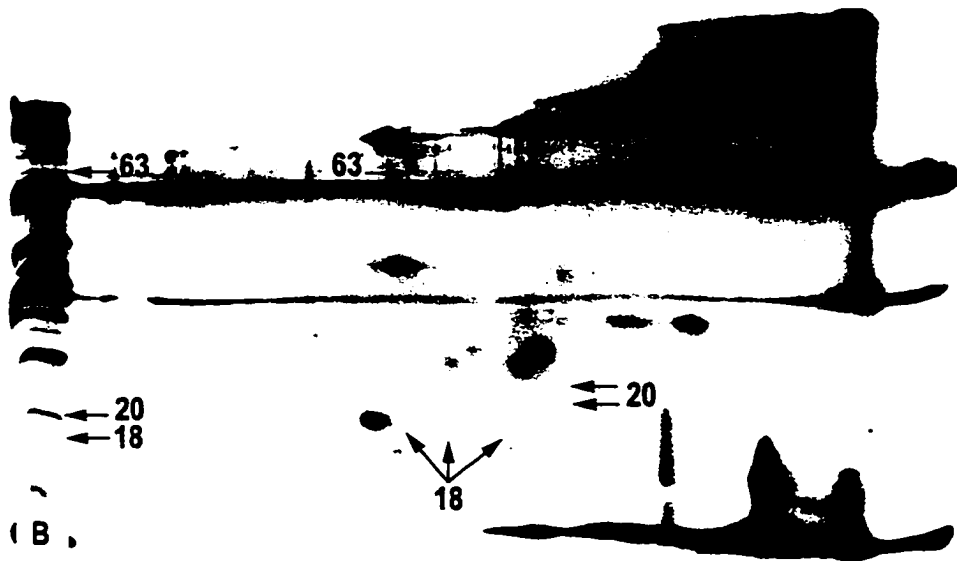
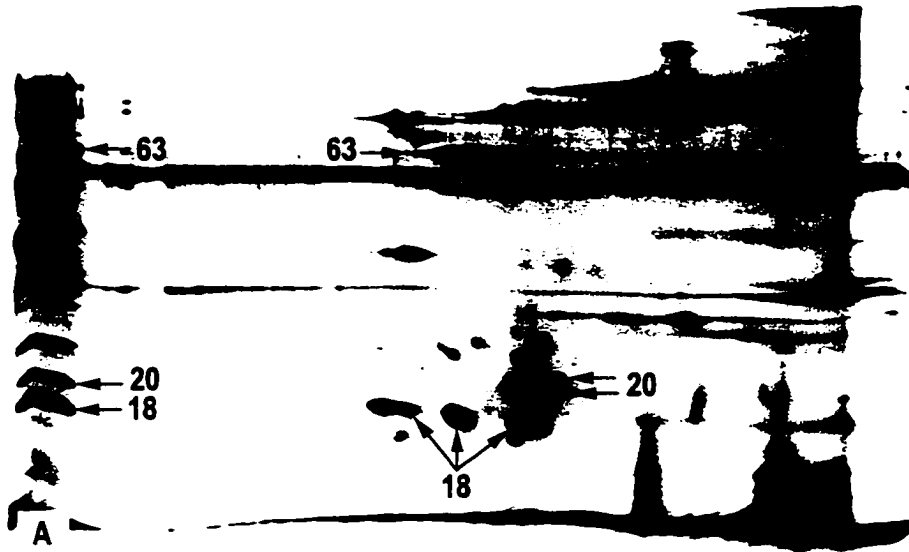


Figure 3.19: Whole cell lysate of induced clone 5d3 separated by 2D-PAGE (A) Total proteins in silver stained gel, (B) Antigenic proteins in western blot probed with GAI-XA and (C) Antigenic proteins in western blot probed with CCS. The 45, 45 and 33 kilodalton cloned antigenic *Edwardsiella ictaluri* proteins expressed by this clone are marked by their molecular weights.



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Figure 3.20: Whole cell lysate of *Edwardsiella ictaluri* separated by 2D-PAGE (A) Antigenic proteins in western blot probe with SAG(pBK) and (B) Antigenic proteins in western blot probed with SAG(4d6). The 63, 20 and 18 kilodalton bands and proteins recognized by antibodies in SAG(pBK) and no longer recognized by SAG (4d6) are marked by their molecular weights.



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Enzyme Assays

Aldolase Assay

Aldolase activity measured in the WCLS of pBK and induced clones 1.4, 5d2 and 5d3 is shown in Units/mL/ μ g in Table 3.12. Results showed that compared to the aldolase activity in control pBK WCL, the 1.4 WCL and 5d2 WCL both had lower aldolase activity, and the 5d3 WCL had higher aldolase activity. The 5d3 WCL had 111% higher aldolase activity than pBK WCL, 590.6% higher aldolase activity than 1.4 WCL and 195.8% higher aldolase activity than 5d2 WCL. These results suggests the cloned fda gene in the 5d3 insert is being expressed.

Table 3.12: Aldolase enzyme activity in clone whole cell lysates (WCL) expressed as Sigma Units per milliliter per microgram of WCL protein (Units/mL/ μ g).

Clone	Units/mL/ μ g
pBK	9.193
1.4	2.801
5d2	6.540
5d3	19.346

Ribose 5-Phosphate Isomerase Assay

Ribose 5-phosphate isomerase (RPI) activity measured in WCLS of pBK and induced clones 1.4, 5d2 and 5d3 is shown in μ mol/min/ μ g in Table 3.13. Results showed the 1.4 WCL and 5d2 WCLs had higher RPIA activity than the control, and the 5d3 WCL had lower RPIA activity compared to RPIA activity in the control pBK WCL. The RPIA activity was 58.7 and 41.2 % higher in 1.4 and 5d2 WCLs respectively compared to RPIA activity in the 5d3 WCL.

Table 3.13: Ribose 5-phosphate isomerase enzyme activity in clone whole cell lysates (WCL) expressed as micromoles of ribulose 5-phosphate formed per minute per microgram of WCL protein (μ mol/min/ μ g).

Clone	Units/mL/ μ g
pBK	1.490
1.4	1.797
5d2	1.599
5d3	1.132

Putative Identification Genes Encoding Antigenic *E. ictaluri* Proteins

Based on combined results of DNA sequence analysis and protein expression and western blotting analysis of clones 4d6, 5d2 and 5d3, some antigenic *E. ictaluri* proteins could be putatively identified.

Antigenic Proteins Encoded and Expressed by Clone 4d6

Protein expression analysis of clone 4d6 showed that three antigenic proteins were being expressed by the *E. ictaluri* insert. The DNA sequence data showed that of the eleven possible ORFs identified, only eight expressed proteins with the same estimated molecular weights as the expressed proteins, and of the eight only four had pI values in the same range as the expressed proteins (Table 3.3). The ORF 4 encoded a putative 54.5 KD *E. ictaluri* protein and was re-designated as the gene *eip55*. The ORFs 3, 6 and 8 encoded a putative 17.8, 18.8 and 19.5 KD protein respectively. These ORFs were re-designated as the genes *eip18*, *eip19* and *eip20*. The *eip55* gene was the only ORF that encoded a putative protein large enough to compare to the 63 KD expressed antigenic protein. The *eip18*, *eip19* and *eip20* genes all encoded putative proteins with molecular weights and pI values comparable to the 18 and 20 KD expressed antigenic proteins.

The PC/Gene analyses of deduced amino acid sequences of *eip18*, *eip19*, *eip20* and *eip55* characterized all four putative proteins as peripheral. The predicted folding type of the *eip55* putative protein was alpha helical (α), *eip18* putative protein was beta pleated sheet (β) and *eip19* and *eip20* putative proteins were both a mix of alpha and beta folding (α/β). Amino acid sequences were analyzed for a variety of motifs using PC/Gene and GCG, with none being identified.

Comparisons of cloned *E. ictaluri* proteins of 4d6 with proteins in an *E. ictaluri* WCL using SAG(4d6) showed the expressed 63, 20 and 18 KD cloned proteins appeared to be the same proteins as a 63, 20 and 18 KD antigenic protein in the *E. ictaluri* WCL. A comparison of encoded and expressed antigenic *E. ictaluri* proteins is shown in Table 3.14. Results suggest both the cloned 4d6 63 KD antigenic protein and the *E. ictaluri* 63 KD antigenic protein are the

same and they may be expressed by the putative *eip55* gene. Results also suggest the cloned 4d6 20 KD antigenic protein and the *E. ictaluri* 20 KD antigenic protein are the same proteins, and the cloned 4d6 18 KD antigenic protein and the *E. ictaluri* 18 KD antigenic protein are the same proteins, and these two proteins may be expressed by two of the three putative genes *eip18*, *eip19* or *eip20*. It could not be determined which of the three genes were expressing which proteins.

Table 3.14: Estimated molecular weights (MW) in kilodaltons and approximated isoelectric points (pI) of putative genes expressing antigenic proteins in the *Edwardsiella ictaluri* insert of clone 4d6 compared with antigenic proteins expressed by clone 4d6 and the corresponding proteins expressed by *E. ictaluri*.

Encoded 4d6			Expressed 4d6		Expressed <i>E. ictaluri</i>	
Gene	MW	pI	MW	pI	MW	pI
<i>eip55</i>	54.5	4.85	63.0	5.5	63.0	5.5
<i>eip20</i>	19.5	5.09	20.0	5.5	20.0	5.5
<i>eip19</i>	18.8	4.78	18.0	5.5	18.0	5.5
<i>eip19</i>	17.8	4.96				

Antigenic Proteins Encoded and Expressed by Clones 1.4, 5d2 and 5d3

Estimated molecular weights and pI values calculated, using PC/Gene, from deduced amino acid sequences of the putative proteins encoded by 1.4, 5d2, and 5d3 inserts were compared with estimated molecular weights and approximated pI values of antigenic proteins that were expressed by clones 1.4, 5d2 and 5d3. These were also compared with estimated molecular weights and pI values calculated from deduced amino acid sequences of the homologous genes encoded in *E. coli*, using the Swiss 2D-service, and, if available, with actual molecular weights and pI values of *E. coli* proteins determined experimentally and reported by the Swiss 2D-service or in the literature. These results are summarized in Table 3.15.

The PC/Gene analysis of deduced amino acid sequences of RPIA and ICIA characterized both proteins as peripheral, with α/β folding. The YGGE protein was characterized as an integral membrane protein, with one transmembrane domain, a secretory signal and α/β folding.

The YGGB protein was characterized as an integral membrane protein, with three transmembrane domains and α/β folding. Aldolase was characterized as an integral membrane protein with α/β folding, though no transmembrane domains were identified.

The ICIA protein and aldolase both have characteristic signatures in their amino acid sequences. The Motifs program of GCG was used to identify and describe these signatures in amino acid sequences deduced from the genes encoded by the 5d23 sequence. The ICIA protein is in the family of bacterial transcriptional regulatory proteins. These proteins bind DNA through a 'helix-turn-helix' motif in their N-terminal section. The ICIA protein is in the subfamily LysR. The LysR signature was identified in the amino acid sequence of *E. ictaluri* ICIA from residues 15 to 51. The class-II aldolases found primarily in bacteria and fungi require a divalent metal ion, usually zinc, for their activity. The signature for these proteins include the region containing two histidine residues involved in zinc binding, and a cluster of acidic residues and glycine located in the C-terminal section of the protein. The zinc binding signature was identified in the amino acid sequence of *E. ictaluri* aldolase from residues 94 to 116, with histidine residues located at positions 107 and 110. The acidic signature was located from residues 166 to 187.

The expressed antigenic proteins were putatively identified based on estimated molecular weights and pI values of encoded and expressed proteins of the *E. ictaluri* inserts of clones 1.4, 5d2 and 5d3 and *E. coli* (Table 3.15), together with results of the enzyme assays (Tables 3.12 and 3.13) and SAG western blots (Table 3.11). Results suggest the 27 KD antigenic protein expressed by clones 1.4 and 5d2 is the RPIA protein, the 35 KD antigenic protein expressed by clones 1.4 and 5d2 is the ICIA protein, and the 33 KD protein expressed by clones 1.4, 5d2 and 5d3 is the YGGE protein. The results suggest the 45.0 KD antigenic protein expressed by clone 5d3 is aldolase since the same separation pattern into two spots was seen in 2D-PAGE gels with both 5d3 and *E. coli*, and because aldolase activity of the 5d3 clone was more than two times higher than pBK or 5d2. The hypothetical YGGB protein did not appear to be expressed by clone 5d3. It could not be determined what genes were expressing the 16 and 6d5 had the same insert, with slight differences in size, in the same orientation behind the

Table 3.15: Estimated molecular weights (MW) in kilodaltons and approximated isoelectric points (pI) of the putative genes encoding antigenic proteins in *Edwardsiella ictaluri* inserts of clones 1.4, 5d2 and 5d3 compared with antigenic proteins expressed by clones 1.4, 5d2 and 5d3, and corresponding proteins encoded and expressed by *Escherichia coli*. Values reported for *E. coli* were obtained using the Swiss 2D-service, unless denoted with a numerical superscript and corresponding reference. No values are given for proteins that were not encoded or expressed. Values that were not determined are denoted with nd.

Encoded 1.4			Expressed 1.4		Encoded 5d2		Expressed 5d2		Encoded 5d3		Expressed 5d3		Encoded <i>E. coli</i>		Expressed <i>E. coli</i>	
Gene	MW	pI	MW	pI	MW	pI	MW	pI	MW	pI	MW	pI	MW	pI	MW	pI
<i>rplA</i>	22.8	5.20	27.0	5.7	22.8	5.20	27.0	5.7	-	-	-	-	22.9	5.20	25.9 27.0 ¹	5.06 -
<i>iciA</i>	33.3	7.69	35.0	≥6.6	33.3	7.69	35.0	≥6.6	-	-	-	-	33.5	6.40	nd	nd
<i>yggE</i>	26.0	7.65	33.0	≥6.6	26.0	7.65	33.0	≥6.6	26.0	7.65	33.0	≥6.6	26.6	6.10	nd	nd
<i>yggB</i>	11.2 (p)	4.23	-	-	-	-	-	-	30.6	9.04	-	-	30.9	7.90	nd	nd
<i>fda</i>	-	-	-	-	-	-	-	-	39.2	5.35	45.0 45.0	5.9 6.6	39.0	5.52	39.8 40.7 42.0 ²	5.55 5.43 -
-	-	-	-	-	-	-	16.0	nd	-	-	19.0	nd	-	-	-	-

¹(Hove-Jensen and Mølgaard 1993)

²(Baldwin, et al. 1978)

vector's promoter. Further analysis of expression of the cloned antigenic *E. ictaluri* proteins showed these three clones all expressed two proteins of 38 and 12 KD. KD antigenic protein of clone 5d2, and the 19 KD antigenic protein of clone 5d3. No ORFs were identified that corresponded to deduced proteins of this size, and the difference in protein expression between clones 1.4 and 5d2 could not be explained. In the SAG western blots, antibody recognition of these two proteins was not eliminated by absorbition.

DISCUSSION

A pool of clones expressing antigenic proteins of *E. ictaluri* was successfully generated by antibody screening of an *E. ictaluri* genomic library in the *E. coli* expression vector XL0LR pBK-CMV (Stratagene). The library was originally screened with GAI-XA, but subsequent screening of WCLs of each individual clone showed most of the cloned antigenic proteins could be identified by *E. ictaluri* antisera generated in either a goat or in catfish.

Restriction enzyme digestion of plasmid DNA of each clone showed the *E. ictaluri* insert sizes ranged from 1.9 to 7.7 Kb. Cloned antigenic *E. ictaluri* proteins could be detected in WCLs of 27 of the 32 positive clones by SDS-PAGE western blotting with GAI-XA and CCS. Based on protein band patterns in SDS-PAGE western blots, the 32 clones were subdivided into eleven groups. Nine clones representing different protein groups were selected for further analysis (Table 3.1).

The antigenic *E. ictaluri* proteins expressed by the nine clones had the same levels of expression both with and without IPTG induction. Partial DNA sequence obtained from each clone showed all but two clones had stop codons located within the first 60 bp of the insert which were in frame with the pBK-CMV IPTG-inducible promoter (Table 3.2). These results indicated that the cloned antigenic proteins being expressed by the clones had their own transcriptional promoters which were recognized by *E. coli* RNA polymerase.

Although the nine clones were selected because they all appeared to represent different patterns of cloned antigenic protein expression, partial DNA sequence results showed several of the clones had the same, similar or overlapping inserts. Clones 1.1, 4d6 and 5d4 all had

unique inserts. Clone 1.1 expressed two proteins of 23.0 and 18.0 KD, clone 5d4 expressed an 18.0 KD protein and clone 4d6 expressed three proteins of 63, 20 and 18 KD. Clones 5d1, 5d8

The partial sequences obtained from inserts of 1.4 and 5d2 indicated these clones had the same insert in opposite orientations, with the 1.4 insert being 207 bp longer at its T7 end than the 5d2 insert. Partial DNA sequences also showed the 1.4 and 5d2 inserts overlapped with the insert of 5d3 (figure 3.10). These three clones all expressed a 33 KD cloned antigenic *E. ictaluri* protein. In addition to the 33 KD protein, clones 1.4 and 5d2 expressed two proteins of 27 and 35 KD. Although clone 5d2 had the same insert as clone 1.4, this clone also expressed a 16 KD antigenic protein that was not expressed by 1.4. In addition to the 33 KD protein, clone 5d3 expressed two proteins of 45 and 19 KD.

Expression of cloned antigenic *E. ictaluri* proteins in the selected clones was further evaluated in 2D-PAGE gels and western blots probed with both GAI-XA and CCS. The SDS-PAGE and 2D-PAGE gel and western blot results are shown in Table 3.10. Not all of the cloned antigenic proteins seen in SDS-PAGE gels and western blots could be seen by 2D-PAGE. The proteins that could not be seen in 2D-PAGE gels may have had pI values outside of the 4.5 to 6.6 pH range of the first dimension IEF gels. Since the IEF tube gels were open-ended, proteins outside of the pH range of the gel might not have run into the gel, or may have run through the gel and out the other end.

Comparisons of SDS-PAGE and 2D-PAGE western blots of each clone probed with GAI-XA and CCS showed some differences in recognition of cloned antigenic proteins between the two antisera (Figures 3.11 and 3.12; Table 3.10). For the clones 4d6, 5d4 and 5d3 recognition was the same with both GAI-XA and CCS. Although clone 1.1 was originally isolated with GAI-XA, antigenic *E. ictaluri* protein bands could only be detected with CCS. In clones 1.4 and 5d2, the 27 KD protein could only be detected with CCS. In clones 5d1, 5d8 and 6d5, which all had the same insert, the 38 KD protein was only recognized by CCS and the 12 KD protein, which appeared to run as a diffuse smear rather than a band, was only recognized by GAI-XA.

The reason the 12 KD band in clones 5d1, 5d8 and 6d5 was only recognized by GAI is not known. Recognition of some proteins by CCS and not GAI-XA is probably due to the fact that

the GAI-XA was heavily absorbed with *E. coli* WCL to remove *E. coli* antibodies. Although CCS had strong cross-reactivity with *E. coli* proteins, it was not absorbed with *E. coli* WCL because the cross-reactivity did not appear to interfere with identification of cloned antigenic *E. ictaluri* proteins. The apparent high level of antigenic cross-reactivity between *E. coli* and *E. ictaluri* suggests the reactivity of goat antiserum to some of the cloned antigenic *E. ictaluri* proteins may have been removed by absorption with *E. coli* WCL.

Partial and complete DNA sequences obtained for each of the nine clones showed homologous gene regions with sequence identities above 50% in inserts of all of the clones except 4d6. Homologous gene regions with sequence identities below 50% were also reported, but may not be biologically relevant. Partial sequences of overlapping inserts of clones 1.4, 5d2 and 5d3 indicated a high level of sequence homology with a contiguous region of the *E. coli* chromosome. Since these three clones appeared to express some cloned proteins that were the same and some that were different, inserts of clones 5d2 and 5d3 were chosen for complete double-strand DNA sequencing. Clone 5d2 was selected over 1.4 because it expressed an estimated 16 KD antigenic protein that clone 1.4 did not. Although the partial DNA sequences obtained from the insert of clone 4d6 did not have sequence homology with any other known sequences, this insert was also chosen for double strand sequencing because it appeared to express three highly antigenic cloned *E. ictaluri* proteins, and it was the only clone that expressed cloned *E. ictaluri* proteins that could be correlated with proteins expressed by *E. ictaluri* cells.

The DNA sequence showed the *E. ictaluri* insert of clone 4d6 was 5727 bp long. Although a total eleven potential ORFs could be identified in the sequence, many of them overlapped and only four ORFs encoded proteins whose deduced amino acid sequences had computed molecular weights and pI values close to antigenic *E. ictaluri* proteins expressed by the clone (Table 3.3). Putative promoter regions could be identified in all four ORFs (Table 3.4). The 4d6 clone expressed three cloned antigenic *E. ictaluri* proteins of 63, 20 and 18 KD with pI values around 5.5. The ORF identified as eip55 was the only ORF that encoded a protein large enough to compare with the expressed 63 KD protein. This putative gene encoded a protein

whose deduced amino acid sequence had a molecular weight of 54.5 KD and a pI of 4.85. The ORFs eip20, eip19 and eip18 encoded proteins whose deduced amino acid sequences had molecular weights of 19.5, 18.8, and 17.8, and pI values of 5.09, 4.78 and 4.96 respectively. These three ORFs all encoded proteins comparable to the 20 and 18 KD protein expressed by the clone. It could not be determined which of these ORFs expressed either the 20 or 18 KD proteins.

Analysis of 2D-PAGE western blots of *E. ictaluri* WCLs probed with SAG showed the three cloned antigenic *E. ictaluri* proteins expressed by clone ei4d6 corresponded with three antigenic proteins expressed by *E. ictaluri* cells (Figure 3.20). The proteins expressed by *E. ictaluri* cells had the same molecular weights, pI values and running patterns as the cloned proteins expressed by 4d6 (Figure 3.17B). These results suggested the 63 KD antigenic proteins expressed by *E. ictaluri* and clone 4d6 were the same, and they were encoded by the putative eip55 gene. Results also suggested the 20 and 18 KD proteins expressed by *E. ictaluri* were the same as the 20 and 18 KD proteins expressed by clone 4d6, and these three proteins were expressed by two of the three putative genes, eip20, eip19 and eip18.

The functions of proteins encoded by the putative genes in the insert of clone 4d6 are not known. None of the putative genes had DNA or deduced amino acid sequence homology with other known genes. Analysis of deduced amino acid sequences of the four putative genes showed no motifs or patterns and none of the proteins were characterized as integral membrane proteins. The only thing that can be said with certainty about the proteins expressed by clone 4d6 is they are antigenic to channel catfish.

The double-strand 6362 bp DNA sequence spanning the region encoded by both 5d2 and 5d3 showed strong homology with a 15,443 bp region of *E. coli*. The *E. coli* region (Accession U28377) encoded the genes and ORFs *serA*, *rpiA*, *iciA*, *sbm*, *ygfD*, ORF_o275, ORF_o492, ORF_f303, *yggE*, *yggA*, *yggB*, *fda*, and *pgk*. The *E. ictaluri* sequence encoded the apparent gene homologs of *E. coli* genes *serA* (partial), *rpiA*, *iciA*, *yggE*, *yggB*, *fda*, and *pgk* (partial). The

E. coli genes between *iciA* and *yggE* were missing in the *E. ictaluri* sequence, as well as gene *yggA*.

The protein encoded by *serA* is D-3-phosphoglycerate dehydrogenase (PDGH; Accession P08328), responsible for the first committed step in the phosphorylated pathway of L-serine biosyntheses (Tobey and Grant 1986). The protein encoded by *rpiA* is ribose 5-phosphate isomerase A (RPIA; Accession P27252), part of the nonoxidative branch of the pentose phosphate pathway (Hove-Jensen and Maigaard 1993). The protein encoded by *iciA* is ICIA (Accession P24194), which is a specific inhibitor of chromosomal initiation of replication *in vitro* (Thony, et al. 1991). The proteins encoded by *yggE* and *yggB* are hypothetical integral membrane proteins, with YGGE (Accession P1168) being 26.6 KD and YGGB being a 30.9 KD (Accession P11666). The *fda* gene encodes fructose 1,6-bisphosphate aldolase (aldolase, Accession P11604) which catalyzes the sixth step in the glycolysis pathway (Alefounder, et al. 1989). The gene *pgk* encodes phosphoglycerate kinase (PGK, Accession P11665), which catalyzes the second step in the second phase of the glycolysis pathway. (Stryer 1988).

The close proximity of *rpiA*, *fda*, and *pgk* in the genome may be related to the functional roles of the enzymes that they encode. Glycolysis is the breakdown of glucose to generate metabolic energy in the form of ATP. Aldolase and PGK are both enzymes in the glycolysis pathway, which occurs in the cell cytosol. Aldolase catalyzes the reversible conversion of D-fructose 1,6 bisphosphate to dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate (Stryer 1988). Phosphoglycerate kinase catalyzes the reversible conversion of 1,3-bisphosphoglycerate and ADP into 3-phosphoglycerate and ATP. In the non-oxidative branch of the pentose phosphate pathway, which is also cytosolic, RPIA catalyzes the reversible conversion of D-ribose 5-phosphate to D-ribulose 5-phosphate. This is the first reaction in the path that ultimately converts Ribose 5-phosphate to fructose 6-phosphate and glyceraldehyde 3-phosphate, both of which are intermediates in the glycolysis pathway (Stryer 1988).

The double-stranded DNA sequences of clones 5d2 and 5d3, as well as T3 and T7 partial sequence obtained from clone 1.4, showed which gene homologs each insert could theoretically

express based on whether or not the genes could be transcribed (Table 3.9). Estimated molecular weights and pI values were computed from deduced amino acid sequences of each gene homolog and compared to *E. ictaluri* protein expression data obtained from SDS-PAGE and 2D-PAGE western blots of each of the three clones. The values obtained for encoded and expressed cloned *E. ictaluri* proteins were compared to computed values of deduced amino acid sequences of encoded *E. coli* genes. Experimentally derived molecular weight and pI values were also available for expressed *E. coli* proteins in some cases (Table 3.15).

The *yggE* gene was the only gene encoded in the inserts of all three clones. Protein expression analysis showed that a 33.0 KD cloned antigenic protein was the only protein expressed by all three clones. To determine if the 33 KD proteins expressed by the three clones were the same protein, or different proteins with the same molecular weights, antibody absorption studies were performed using SAG. Results showed the antibodies that recognized the 33 KD protein were the same for all three clones, indicating the 33 KD protein was the same. Since the 33 KD protein was the only one expressed by all three clones, and the *yggE* gene homolog was the only gene encoded by all three clones, the 33 KD protein was putatively identified as YGGE. The molecular weight computed from the deduced amino acid sequence encoded by the *yggE* gene is 26.0 KD for *E. ictaluri* and 26.6 KD for *E. coli*. The molecular weight of the actual expressed *E. coli* YGGE has not been determined because the protein is hypothetical, based only on identification of the ORF. The computed pI value of encoded YGGE is 7.65 for *E. ictaluri* and 6.1 for *E. coli*. The expressed *E. ictaluri* YGGE ran at the edge of the 2D-PAGE gel, suggesting the pI value of the protein was close to the upper range of the pH gradient of the first-dimension IEF gel. The pI value of this protein was therefore approximated to be ≥ 6.6 . The putative YGGE protein of *E. ictaluri* was characterized as an integral membrane protein with one transmembrane domain, a secretory signal and α/β folding that was antigenic to channel catfish.

The *rpiA* and *iciA* genes were only encoded in the inserts of clones 1.4 and 5d2. Protein expression analysis showed these clones expressed two proteins of 35.0 and 27 KD that were not expressed by clone 5d3. The 27 KD protein could only be detected in 2D-PAGE western

blots probed with CCS. Antibody absorption studies using SAG showed the antibodies that recognized the 35 and 27 KD proteins were the same for both clones, indicating the proteins were the same (Table 3.11). The molecular weight and pI values computed from the deduced amino acid sequence encoded by the *rpiA* gene were 22.8 KD, pI 5.2 for *E. ictaluri* and 22.9 KD, pI 5.2 for *E. coli*. The molecular weight of expressed *E. coli* RPIA was reported to be 25.9 to 27.0 KD, pI 5.06. The molecular weight and pI values computed from the deduced amino acid sequence encoded by the *iciA* gene were 33.3 KD, pI 7.69 for *E. ictaluri* and 33.5 KD, pI 6.4 for *E. coli*. The pI value of expressed 27 KD protein was approximated to be 5.2. Since the expressed 35 KD protein ran right at the edge of the 2D-PAGE gel, its pI value was approximated to be ≥ 6.6 . Based on the molecular weights and pI values of expressed and encoded *E. ictaluri* proteins of clones 1.4 and 5d2, and expressed and encoded *E. coli* proteins, the 27 KD cloned *E. ictaluri* protein was putatively identified as RPIA and the 35 KD protein was putatively identified as ICIA. The putative *E. ictaluri* RPIA and ICIA are characterized as being peripheral with α/β folding. In other organisms, the RPIA is located in the cytosol (Stryer 1988). Since the ICIA is a DNA binding protein, it is probably also located in the cytosol.

In an effort to confirm RPIA was being expressed by clones 1.4 and 5d2, an enzyme assay was performed which measured the conversion of ribose 5-phosphate to ribulose 5-phosphate (Hove-Jensen and Maigaard 1993). The RPIA activity was measured in WCLs of clones 1.4, 5d2, 5d3 with pBK measured as a control. The results showed that RPIA activity was higher in both 1.4 and 5d2 WCLs than in 5d3 and the control, with the lowest activity seen in the 5d3 WCL (Table 3.13). This is further evidence that RPIA was expressed by clones 1.4 and 5d2, though the differences in levels of expression were not great as might be expected. This may be due to the fact that, although multiple copies of the *rpiA* gene were present in the 1.4 and 5d2 clones, expression of the gene was regulated because it was transcribed from its own promoter, rather than the IPTG-inducible pBK-CMV promoter. Since the 1.4 and 5d2 clones were grown in LB broth, which contains glucose, products of the non-oxidative pentose phosphate pathway would not have been needed. Glucose 6-phosphate, the first breakdown product of glucose in

the glycolysis pathway (Stryer 1988), has been shown to have an inhibitory effect on RPIA concentrations produced by *E. coli* K-12 cells (Essenberg and Cooper 1975).

The ICIA protein is a homodimer that binds to three 13-mers in the origin (oriC) of *E. coli* to block initiation of replication by the DnaA initiator protein. The ICIA protein belongs to the LysR family of prokaryotic regulators which act as transcriptional activators. These proteins can also regulate their own expression and are identified by a potential helix-turn-helix signature motif located in their N-terminal domain (Thony, et al. 1991). A gel shift assay was used to demonstrate over-expression of a DNA binding protein *in vitro* for cloned *E. coli* ICIA (Thony, et al. 1991). The gel shift assay was not employed in this study, however the helix-turn helix motif was identified in the deduced amino acid sequence of ICIA encoded by the 1.4 and 5d2 inserts. The 1.4 and 5d2 clones did not exhibit any discernable difference in growth from the pBK or any of the other clones. This is consistent with what was observed with the cloned *E. coli* ICIA, which reached the same culture densities as the cloning vector without ICIA, although a considerable lag in growth was observed (Thony, et al. 1991).

The complete *yggB* gene was only encoded in the insert of pBK-ei5d3. The inserts of pBKei1.4 and 5d2 had a partial ORF of the *yggB* gene. Protein expression analysis showed clone 5d3 expressed two proteins of 33 and 45 KD. The 33 KD protein had been putatively identified as YGGE based on its expression in all three clones. The molecular weight and pI values computed from the deduced amino acid sequence encoded by the complete *yggB* gene were 30.6 KD, pI 9.04 for *E. ictaluri* and 30.9 KD, pI 7.90 for *E. coli*. The molecular weight of the actual expressed *E. coli* YGGB has not been determined because the protein is hypothetical, based only on identification of the ORF. The partial *yggB* could not be transcribed in clone 5d2 because the promoter region and N-terminal region of the gene were missing. The partial *yggB* gene was in frame with the pBK-CMV IPTG-inducible promotor of clone 1.4. The molecular weight and pI value of the partial fusion protein were computed from the deduced amino acid sequence encoded by the partial *yggB* gene to be 11.2 KD with a pI of 4.23. In the fusion-protein, an 88 bp region of the β -galactosidase gene would be fused with the partial *yggB* gene

resulting in a protein of 14.4 KD, the pI value of the fusion protein was not determined. Protein expression analysis showed the encoded 30.6 KD protein of clone 5d3 was either not expressed or was not antigenic. The protein was not detected in SDS-PAGE and 2D-PAGE gels and western blots. Since the computed pI value was out of range of the first-dimension IEF tube gels, the protein may not have run in the 2D-PAGE gel. A 14.4 KD protein corresponding to the encoded fusion protein of clone 1.4 also did not appear to be expressed. These results indicate the hypothetical *yggB* gene may not be an actual expressed gene.

The *fda* gene was only encoded by clone 5d3. Protein expression analysis showed this clone expressed a 45 KD protein that was not expressed by clones 1.4 or 5d2. The molecular weight and pI values computed from the deduced amino acid sequence encoded by the *fda* gene were 39.2 KD, pI 5.35 for *E. ictaluri* and 39.0 KD, pI 5.52 for *E. coli*, pI 5.43 to 5.55. The molecular weight of expressed *E. coli* aldolase was reported to be 39.8 to 42.0 KD. The 2D-PAGE results reported for expressed *E. coli* aldolase show the protein separates into two spots with pI values of 5.43 and 5.55 (Swiss 2D-Service). The cloned 45 KD protein expressed by clone 5d3 separated into two spots in 2D-PAGE gels with approximated pI values of 5.9 and 6.6. Based on molecular weights and pI values of the expressed and encoded cloned *E. ictaluri* protein of clone 5d3 and the expressed and encoded *E. coli* protein, the 45 KD cloned *E. ictaluri* protein was putatively identified as aldolase. The putative *E. ictaluri* aldolase was characterized as an integral membrane protein with α/β folding, although no transmembrane domains were identified. Since glycolysis occurs in the cell cytoplasm, and there were no transmembrane regions identified in the protein, the characterization as an integral membrane protein is probably in error.

The fructose 1,6-bisphosphate aldolases are divided into two classes based on their molecular and catalytic properties. Class I aldolases are found in animals, plants and green algae. Class II aldolases occur in eukaryotic fungi and green algae and prokaryotic bacteria and blue-green algae (Baldwin, et al. 1978). Certain photosynthetic organisms including *E. gracilis*, *Chlamydomonas mundana* and *C. reinhardtii* can produce either class I or class II aldolase

depending on growth conditions (Stribling and Perham 1973). Class I aldolases are typically homotetramers with a subunit molecular weight of 40 KD. Class II aldolases are normally homodimers with a subunit molecular weight of 30 to 40 KD. Class II aldolases are strongly inhibited by metal chelating agents such as EDTA. A divalent metal ion is required for the formation of the active metalloprotein complex. The metal ion used by the class II aldolases is usually zinc, but may also be cobalt, iron, nickel or manganese (Stribling and Perham 1973). The *E. coli* class II aldolase contains two zinc ions per enzyme dimer (Berry and Marshall 1993).

Class II aldolases are identified by a signature motif consisting of two conserved regions. The signature includes the region containing the two histidine residues which are involved in zinc binding, and a cluster of acidic residues and glycine located in the C-terminal region of the protein. In *E. coli*, site directed mutagenesis has shown that histidine residues His-108 and His-111 are directly responsible for the tight binding of zinc (Berry and Marshall 1993). The zinc binding region was identified in the deduced amino acid sequence encoded by cloned *E. ictaluri fda*. The region spans the residues 94 to 116, and includes histidine residues His-107 and His-110. The acidic signature was located from residues 166 to 187.

To confirm that aldolase was being expressed by clone 5d3, a commercially available enzyme assay (Sigma 752) was performed which measured conversion of fructose 1,6-bisphosphate to hydrazone. Aldolase activity was measured in WCLs of clones 1.4, 5d2, 5d3 with pBK measured as a control. Results showed aldolase was 2-7 fold higher in the 5d3 than in 1.4, 5d2 and the control (Table 3.12). This is further evidence aldolase was expressed in 5d3, though the differences in the levels of expression were not great as might be expected. As with RPIA, this may be due to the fact that, although multiple copies of the *fda* gene were present in the 5d3 clone, expression of the gene was regulated because it was transcribed from its own promoter, rather than an inducible promoter.

Alternatively, the lower than expected levels may be due to the nature of the aldolase assay, which was designed for use on serum. With this assay, aldolase converts fructose 1,6-bisphosphate into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. Triose

phosphate isomerase (TPI) present in serum then catalyzes almost complete conversion of glyceraldehyde 3-phosphate to dihydroxyacetone phosphate. Further reactions irreversibly convert dihydroxyacetone phosphate into hydrazone which produces a color reaction that is proportional to aldolase activity. Unlike serum, bacterial WCLs may not contain TPI, or levels of TPI sufficient for complete conversion of the glyceraldehyde 3-phosphate into dihydroxyacetone phosphate. The color reaction which measures aldolase activity is based solely on the amount of dihydroxyacetone phosphate formed; therefore, if the glyceraldehyde 3-phosphate is not completely converted, the assay will measure a lower level of aldolase activity than may actually be present.

Class II aldolase appears to be highly conserved among prokaryotes, fungi and blue-green algae (Alefounder, et al. 1989). Although the highest percent sequence identity (82.1%) was seen with *fda* of *E. coli* (Table 3.6), cloned *E. ictaluri fda* also had sequence identities with a number of other organisms. These included the bacteria *H. influenza*, the blue-green algae *E. gracilis*, and the fungi *N. crassa*, *Sc. pombe*, and *Sa. cerevisiae* (Table 3.7). Cloned *E. ictaluri rpiA*, in addition to sequence homology with *E. coli* (75%), also had homology with *rpiA* of *A. salmonicida* (Table 3.7). It is interesting to note that *A. salmonicida* is also a fish pathogen.

Two antigenic proteins that were expressed by clones 5d2 and 5d3 could not be identified with encoded genes. It could not be determined what genes were expressing the 16 KD antigenic protein of clone 5d2, and the 19 KD antigenic protein of clone 5d3. In SAG western blots, antibody recognition of these two proteins was not eliminated by absorption. No ORFs were identified that corresponded to deduced proteins of this size, and the difference in protein expression between the 1.4 and 5d2 clones could not be explained. Clone 1.4 could have theoretically expressed a partial YGGB fusion protein that was in frame with the IPTG-inducible pBK-CMV promoter, but this was not observed. This could not occur in clone 5d2 due to the opposite orientation of the insert. Since there was no evidence that the hypothetical 30.6 KD YGGB protein was expressed, the possibility that the start codon of *yggB* was mis-identified, resulting in a smaller protein, was investigated. The calculated molecular weights of proteins

encoded from the first two methionine residues located downstream from the methionine start codon of *E. ictaluri* *yggB* were too large and too small to account for 19.0 KD antigenic protein expressed by clone 5d3.

This study has resulted in the putative identification of a number of antigenic *E. ictaluri* proteins. One is a 63 KD protein encoded by the *E. ictaluri* gene *eip55*. Two other proteins of 20 and 18 KD were determined to be encoded by two of three genes named *eip20*, *eip19* and *eip18*. The functions of these putative antigenic proteins are unknown since the nucleic acid and amino acid sequences of these genes and encoded proteins had no sequence homologies with any other known sequences in the database. The enzymes RPIA and aldolase, the transcriptional regulator ICIA, as well as the putative YGGE integral membrane protein, which has an unknown function, were also identified as putative antigenic *E. ictaluri* proteins.

Identification of these antigenic proteins was based on DNA sequence analysis and characterization of encoded and expressed proteins of each clone. When it was possible, enzyme assays were utilized to demonstrate expression of encoded proteins. These identifications are putative however, because most of the evidence used to identify the proteins was indirect. Since the clones all contained *E. ictaluri* inserts that were large enough to encode and express more than one protein, much of the identification was by the process of elimination. The antigenic protein expression would need to be evaluated for each individual, subcloned gene in order to determine with certainty that the antigenic proteins are encoded by genes they are putatively identified with.

Using 2D-PAGE western blotting, this study also showed anti-*E. ictaluri* antibodies in CCS cross-reacted with a very high number of *E. coli* proteins (Figure 3.13C). Control normal catfish serum, which had no antibodies against *E. ictaluri*, was shown to have no cross-reactivity with *E. coli*. Cross-reactivity between *E. ictaluri* and *E. coli* has been reported previously and was assumed to be due to cross-reactivity between a conserved LPS core antigen (Tyler and Klesius 1994c; Tyler and Klesius 1994b). In this study, there was no discernable difference in the level

of antibody recognition after absorption of CCS with *E. ictaluri* LPS. This indicates the antibodies were primarily against proteins rather than a conserved LPS core antigen.

To my knowledge, this study represents the first attempt to identify antigenic *E. ictaluri* antigens by more than their molecular weight or location in the cell. The antigenic *E. ictaluri* proteins that were putatively identified were enzymes and transcriptional regulators. These are structural proteins with housekeeping functions, as opposed to virulence factors. It should not be assumed, however, that this precludes them from being protective antigens. Protective T cell responses against cross-reactive bacterial antigens have been frequently observed. These cross-reactive antigens are conserved proteins shared by a number of bacteria (Kaufmann 1993). The high antibody cross-reactivity and conservation of the proteins between *E. coli* and *E. ictaluri*, as well as other bacteria, leaves open the possibility that the putatively identified cloned *E. ictaluri* proteins are T cell antigens. The identification of potential protective T cell antigens may be required for the development of a successful ESC vaccine since vaccines made up of killed *E. ictaluri* bacterins have been unsuccessful. The failure of these vaccines is likely due to the fact that killed bacterins generally only elicit a humoral immune response unless they are administered with an adjuvant. This suggests that a cell-mediated immune response is a necessary component of the protective immune response of channel catfish.

CHAPTER 4: VACCINATION OF CHANNEL CATFISH WITH CLONED ANTIGENIC PROTEINS OF *EDWARDSIELLA ICTALURI* IN AN *ESCHERICHIA COLI* EXPRESSION VECTOR AND *E. ICTALURI* GROWN IN RICH AND MINIMAL MEDIA

The protective capabilities of 14 cloned antigenic *Edwardsiella ictaluri* proteins expressed by six *Escherichia coli* clones and *E. ictaluri* grown in rich and minimal media were evaluated in vaccination trials measuring protection from challenge. Results showed the percent mortality was significantly lower in all of the vaccine treatments compared to the unvaccinated control. Levels of protection, measured as relative percent survival (RPS) were higher for *E. ictaluri* grown in minimal media (RPS 78.3) than for *E. ictaluri* grown in rich media (RPS 57.9), though no statistical difference was detected due to variation between replicate tanks. Surprisingly, one of the highest RPS values was seen with the control treatment of the *E. coli* host strain of the cloning vector without an *E. ictaluri* insert. Since protective capabilities of the *E. coli* alone were so high, protective capabilities of the clones expressing antigenic *E. ictaluri* proteins could not be ascertained. Protective capabilities of *E. coli* suggest cross-reactive vaccination may be an additional vaccine strategy to investigate in terms of developing a successful ESC vaccine.

INTRODUCTION

Edwardsiella ictaluri, causative agent of enteric septicemia of catfish (ESC), is the primary bacterial pathogen of commercially produced channel catfish (*Ictalurus punctatus*) in the Southeastern United States. Mortality losses and treatment costs are estimated to be 19 million dollars annually (Thune, et al. 1997b). Current treatment is with antibiotics, however, increases in the number of antibiotic-resistant *E. ictaluri* strains are reducing the effectiveness of these drugs (Thune and Johnson 1992).

It is generally assumed that fish surviving an *E. ictaluri* infection are resistant to ESC if they recover (Klesius and Horst 1991). *Edwardsiella ictaluri* is considered a good candidate for vaccine development because it has a very high serologic homogeneity (Plumb 1988; Plumb and Vinitnantharat 1989; Bertolini, et al. 1990). Like other fish pathogens, vaccines against *E. ictaluri* initially focused on using inactivated bacterial preparations without any understanding of factors important in eliciting a protective immune response against ESC. Unfortunately, this

approach has not been successful, and efforts are now being made to identify important antigens. In addition, more recent efforts have recognized the need to stimulate a cell-mediated immune response.

Since specific antibodies, activated macrophages and helper T cells may all be components of the protective immune response against *E. ictaluri* (Scott, et al. 1985; Sheldon and Blazer 1991; Waterstrat, et al. 1991; Vinitnantharat and Plumb 1993; Wise, et al. 1993; Shoemaker, et al. 1997), a vaccine designed to elicit these responses should provide protective immunity against ESC.

One approach to developing a vaccine of this type is to identify antigens of *E. ictaluri* that elicit the protective humoral and cellular responses. An approach to identifying protective antigens is to first identify candidate bacterial proteins antigenic to the host, that is, proteins reacting with host antibodies following recovery from infection. Although an antibody response does not necessarily correspond with protective immunity, identification of antigenic proteins may narrow the field of possible protective antigens for further studies. The next step is to characterize some of these candidate proteins in order to determine what types of proteins are antigenic to channel catfish. The final step is to ascertain the protective capabilities of the antigens. Success of this depends on presenting antigens in the appropriate manner.

The first two steps of this process have been completed. A pool of clones expressing antigenic *E. ictaluri* proteins was generated by antibody screening of an *E. ictaluri* genomic library in the *Escherichia coli* expression vector XL0LR pBK-CMV (Stratagene, La Jolla California, USA). Representative clones from this pool were characterized by DNA sequencing and protein expression analysis. Those results are reported and discussed in Chapter 3. The final step in the process of characterizing these clones is to ascertain protective capabilities of cloned antigenic *E. ictaluri* proteins. This can be done in vaccine trials by vaccinating fish with individual cloned proteins in the form of subunit vaccines. Subunit vaccines can be produced in *E. coli* or yeast vectors designed to express large amounts of cloned proteins. Expressed cloned proteins

can then be used as vaccines either in cell lysates of the recombinant expression system or as purified proteins (Munn 1994).

Previous research (Chapter 1) showed expression of antigenic *E. ictaluri* proteins was effected by culture medium, with an additional protein being expressed in a defined minimal media broth (MM19; Collins and Thune 1996)) but not in a rich brain-heart infusion broth (BHI). This protein was recognized by antibodies in sera of catfish recovered from ESC infections, demonstrating it was expressed during an *E. ictaluri* infection. Since proteins that are only expressed in MM19 may be protective antigens, vaccine preparations of *E. ictaluri* cells grown in MM19 may be better at eliciting a protective immune response than cells grown in rich media.

The purposes of this research were two-fold. The first purpose was to evaluate protective capabilities of cloned antigenic proteins of *E. ictaluri* described and characterized previously using subunit vaccines of cloned proteins in whole cell lysates (WCLs) of the *E. coli* expression vector XL0LR pBK-CMV. The second goal was to determine if there were differences in protective capabilities of *E. ictaluri* WCLs prepared from cells grown in BHI or MM19 media.

MATERIALS AND METHODS

Bacterial Strains, Media and Antibiotics

Escherichia coli strain XL0LR pBK-CMV was grown at 37 °C on Luria-Bertani (LB) media with kanamycin (Kan, 50 µg/ml; Sigma Chemical Co., St. Louis, Missouri, USA) selection to maintain the pBK-CMV plasmid (Sambrook, et al. 1989). Fusion protein expression was induced in XL0LR pBK-CMV clones containing inserts of *E. ictaluri* genomic DNA (XL0LR pBK-ei) by adding IPTG (isopropylthio-β-D-galactose; Amresco, Solon, Ohio, USA) to 2 hour LB broth cultures for a final concentration of 1 mM IPTG, then growing overnight. *Edwardsiella ictaluri* virulent strain 93-146 was grown on tryptic-soy agar with 5% sheep blood (TSA II) plates, or in BHI or MM19 broth at 25-28 °C.

***E. coli* XLOR pBK-ei Clones Expressing Antigenic *E. ictaluri* Proteins**

The *E. coli* clones XLOR pBK-ei1.1(1.1), XLOR pBK-ei1.4 (1.4), XLOR pBK-ei5d1 (5d1), XLOR pBK-ei5d2 (5d2), XLOR pBK-ei5d3 (5d3), XLOR pBK-ei5d4 (5d4), XLOR pBK-ei5d8 (5d8), and XLOR pBK-ei6d5 (6d5) were described previously in Chapter 3. These six clones all expressed cloned *E. ictaluri* proteins recognized by *E. ictaluri* antiserum generated in both a goat and in catfish.

Bacterial Cell Preparations

Edwardsiella ictaluri and induced *E. coli* XLOR pBK-CMV (pBK) and clones with *E. ictaluri* inserts were harvested by centrifugation at 3800 x gravity for 15 min at 4 °C and washed three times in phosphate buffered saline, pH 7.3. The supernatant was discarded and cell pellets were either processed immediately, or stored at -20 °C until processing. To process, cells were resuspended in sterile distilled deionized (DDI) water to a standardized volume of 0.1 mL of cell pellet per mL and sonicated at 40 W at 50% duty cycle for approximately 8 min, or until cell suspensions changed from milky to clear. Sonicated whole cell lysate (WCL) was allowed to sit for 1 hr at 4 °C and 10% sodium ethylmercurithiosalicylate (thimerosal; Sigma) was added to the supernatant to achieve a final concentration of 0.01% thimerosal. The supernatant was then stored at -20 °C until use.

In the second vaccine trial, WCLs were thawed four days prior to use, brought to a final concentration of 0.03% formalin and rocked on an aliquot mixer at 4 °C overnight. To confirm sterility, thioglycolate broth tubes were inoculated with each formalized WCL and incubated at 28 °C for *E. ictaluri* WCLs and 37 °C for *E. coli* WCLs. Formalized *E. ictaluri* WCLs were also streaked onto plates. Cultures were checked for growth after 24 and 48 hours. Formalized WCLs were stored at 4 °C until use.

Specific Pathogen free (SPF) Channel Catfish

Channel catfish egg masses were obtained from commercial producers with no history of ESC outbreaks and disinfected with 100 ppm free iodine. The eggs were hatched in closed recirculating systems in the specific pathogen free (SPF) laboratory at the School of Veterinary

Medicine, LSU, and maintained to the juvenile stage. Experimental SPF fish were transferred to a separate laboratory into 20 L flow-through tanks supplied with dechlorinated municipal water maintained at 25 ± 1 °C at a flow-rate of 300–400 mL per minute. Fish were fed commercial catfish feed *ad libitum* every other day during acclimation and through-out experiments.

Vaccination Trial One (VT1)

Acclimation

Experimental SPF fish were transferred into 20 L flow-through tanks stocked at a density of 30 fish per tank. Fish were acclimated to the flow-through system for 24 days, then impartially redistributed into 18 tanks with 12 fish per tank and allowed to acclimate for 40 more days before being vaccinated.

Vaccination and Boost

On the day of vaccination, tanks were randomly divided into six treatment groups with three tanks per treatment. Fish (10.2 ± 0.60 g) were anesthetized with tricaine methanesulfonate (MS-222; Argent Chemical Laboratories, Redmond Washington, USA), and individually injected intramuscularly (IM) with 0.1 mL of the assigned vaccine treatment. The six vaccine treatments included two saline controls, WCLs of *E. ictaluri* grown in BHI and MM19, WCL of induced pBK, and a mixture containing equal volumes of WCLs of each of the six induced clones. The WCLs used were not formalized. Booster injections of the same treatments were administered 27 days after initial vaccination.

Challenge by Experimental Infection

Thirty days following booster injections, fish in all treatments except a control were challenged by experimental infection with *E. ictaluri* using immersion exposure. For experimental infections 5.0 ml cultures of BHI were inoculated with *E. ictaluri* strain 93-146 from stock cultures stored at -80 °C, then incubated overnight at 28 °C on a tissue culture rotator. Two mL of overnight culture was added to 2 L Erlenmeyer flasks containing 750 mL sterile BHI with a drop of antifoam 289 (Sigma), and incubated on a shaker overnight at 28 °C and 200 revolutions per

minute. Overnight flask cultures were combined, then streaked on plates to confirm purity. Cell density of the combined culture was measured on a spectrophotometer at OD₆₀₀.

For immersion exposure, water flow was stopped and 200 mL of the combined *E. ictaluri* culture was added to each treatment tank along with three drops of antifoam. As a control, a saline injected treatment group was exposed to 200 mL sterile BHI broth with three drops antifoam. Water flow was resumed following a 15 minute exposure. Mortalities were recorded every 24 hours post-exposure for 68 days. To confirm *E. ictaluri* infections, a necropsy was performed on each mortality and tissue samples aseptically collected from the trunk kidney and head lesions were plated and incubated overnight at 30 °C.

Vaccination Trial Two (VT2)

Acclimation

Experimental SPF fish previously acclimated to a flow-through system in the SPF laboratory were transferred into 45, 20 L flow-through tanks stocked at a density of 10-11 fish per tank. Fish were acclimated for 32 days, then impartially redistributed so 36 of the 45 tanks held 11 fish each. Fish were vaccinated 2 days following redistribution.

Vaccination and Boost

On the day of vaccination, the 36 tanks were randomly divided into twelve treatment groups with three tanks per treatment. Fish (31.5 ± 3.49 g) were anesthetized with MS-222 and individually injected IM with 0.1 mL of the assigned vaccine treatment. The twelve vaccine treatments included two saline controls, WCLs of *E. ictaluri* grown in BHI and MM19, WCLs of induced pBK and clones 1.1, 4d6, 5d2, 5d3, 5d4 and 6d5, and a mixture containing equal volumes of WCLs of each of the six induced clones. The WCLs used had been formalized and confirmed sterile. Booster injections of the same treatments were administered 28 days after initial vaccination.

Challenge by Experimental Infection

Twenty-eight days following booster injections, fish in all treatments except a saline injected control were challenged by experimental infection with *E. ictaluri* using immersion

exposure. For experimental infections, *E. ictaluri* cultures were grown in the same manner as described for VT1, with the exception that the 5.0 ml cultures of BHI were inoculated with single colonies from a plate of *E. ictaluri* strain 93-146 that had been passed twice through fish to confirm virulence. Overnight flask cultures were combined, then streaked on plates to confirm purity. Cell density of the combined culture was measured on a spectrophotometer at OD₆₀₀. Cell numbers of the combined culture were also quantified by serially diluting in triplicate in sterile saline, and dropping 20 µL of each dilution onto plates. Plates were allowed to dry, then inverted and incubated at 28 °C for 48 hours.

For immersion exposure of each treatment tank, water flow was stopped, tank volumes were lowered to 10 L and 200 mL of combined *E. ictaluri* culture was added. As a control, a saline injected treatment group was exposed to 200 mL sterile BHI broth. Water flow was resumed immediately after the culture was added. Mortalities were recorded every 24 hours post-exposure until there were no mortalities for seven consecutive days. *Edwardsiella ictaluri* infections were confirmed as described for VT1.

Statistical Analyses

Cumulative daily mortality for each tank was normalized with an arcsine squareroot transformation (Neter, et al. 1990), and analyzed by analysis of variance (ANOVA) followed by Tukey's multiple comparison at a significance level of $\alpha = 0.05$ using the General Linear Models Procedure in SAS Version 6.12 (SAS, Cary North Carolina, USA). Relative percent survival (RPS) was calculated using the formula $RPS = (100 \times [\%mortality\ treatment + \%mortality\ controls])$ as described by Amend (1981).

RESULTS

Vaccine Trial One

Due to shortages in the amount of vaccine prepared, there was not enough to inject all 12 fish in each tank of a treatment. As many fish as possible were vaccinated which resulted in a variable number of fish per tank, with numbers ranging from 11 to 12. Feeding activity remained normal and there were no mortalities following vaccination with saline or *E. coli* WCLs,

Mortalities did occur in treatment tanks vaccinated with *E. ictaluri* WCLs, with all of the fish dying in the *E. ictaluri* BHI treatment group, and all but three fish dying in the *E. ictaluri* MM19 treatment group. As a result, these treatment groups were eliminated from the experiment. No mortalities occurred in the remaining treatment groups following booster injections.

Fish were challenged with 2.9×10^{11} *E. ictaluri* cells per treatment tank. Mortalities that occurred following challenge all cultured positive for *E. ictaluri* and had internal or external lesions consistent with either acute or chronic ESC. Mortality results are shown in Table 4.1. Mortalities occurred from day 7 post-challenge through day 68, with the highest mortalities on day 10 and day 40 post-challenge with 3 mortalities both days. Mortalities occurring from day 16 post-challenge had "hole-in-the-head" lesions characteristic of chronic ESC. Results showed that, compared to the challenged saline control treatment, the percent mortality was significantly lower only in the unchallenged saline control treatment. Although RPS values were 80.0 and 79.1 and the percent mortality was lower in vaccination treatments they were not significantly different from the unvaccinated control treatment ($0.05 > P > 0.10$).

Vaccine Trial Two

Due to shortages in the amount of vaccine prepared, there was not enough to inject all 11 fish in each tank of a treatment. As many fish as possible were vaccinated which resulted in a variable number of fish per tank, with numbers ranging from 9 to 11. Feeding activity remained normal and there were no mortalities following vaccination or booster injections.

Fish were challenged with 1×10^{12} cells per treatment tank. Mortalities that occurred following challenge all cultured positive for *E. ictaluri* and had internal or external lesions consistent with acute ESC. Mortality results are shown in Table 4.2. Mortalities occurred from day 6 to day 18 post-challenge, peaking at day 11 with 21 mortalities. Results showed that, compared to the challenged saline control treatment, the percent mortality was significantly lower in the unchallenged saline control treatment, as well as in all of the vaccine treatment groups. Percent survival was not significantly different between any vaccine treatment groups,

Table 4.1: Results from vaccination trial one. The saline vaccinated control was not challenged (nc) with *Edwardsiella ictaluri*. Standard error is denoted by SE. Means with the same letters are not significantly different.

Treatment	Replicate	Total # Fish	# Fish Dead	% Mortality	% Mortality (mean \pm SE)	RPS ¹
Saline, nc	1	12	0	0	0 ^B	
	2	12	0	0		
	3	12	0	0		
Saline	1	12	5	41.7	27.8 \pm 0.07 ^A	
	2	12	2	16.7		
	3	12	3	25.0		
pBK	1	12	2	16.7	5.6 \pm 5.56 ^{AB}	80.0
	2	11	0	0		
	3	11	0	0		
Clones	1	12	1	8.3	5.8 \pm 2.91 ^{AB}	79.1
	2	11	1	9.1		
	3	11	0	0		

¹RPS = Relative percent survival = 100 x [1 - (% mortality vaccinated + %mortality controls)]

although RPS was lowest (57.9) in the treatment group vaccinated with *E. ictaluri* (BHI) and highest (90.5) in the treatment group vaccinated with clone 1.1 WCL.

DISCUSSION

In VT1, fish in the treatment groups vaccinated with *E. ictaluri* BHI and MM19 WCLs died from *E. ictaluri* infections due to viable cells in the WCL preparations. Consequently, the vaccine treatments for VT2 were formalized and checked for sterility before being administered to fish. Mortalities in VT1 were primarily chronic, and total mortality was only 27.8% in the unvaccinated control group. Optimal potency testing of fish vaccines requires a minimum of 60% mortality in non-vaccinated challenged controls (Amend 1981; Ellis 1988a), though some researchers suggest greater than 80% mortality (Ward 1982). This is to assure there are a sufficient number of susceptible fish in the population to allow data analysis and to assume an adequate level of challenge, without overwhelming any protective immunity (Amend 1981). Amend (Amend 1981)

Table 4.2: Results from vaccination trial two. The saline vaccinated control was not challenged (nc) with *Edwardsiella ictaluri*. Standard error is denoted by SE. Means with the same letters are not significantly different.

Treatment	Replicate	Total # Fish	# Fish Dead	% Mortality	% Mortality (mean \pm SE)	RPS ¹
Saline, nc	1	11	0	0	0 ^C	
	2	11	0	0		
	3	11	0	0		
Saline	1	11	11	100.0	87.9 \pm 8.02 ^A	
	2	11	10	90.9		
	3	11	8	72.7		
<i>E. ictaluri</i> (BHI)	1	11	8	72.7	37.0 \pm 17.9 ^B	57.9
	2	11	2	18.2		
	3	10	2	20.0		
<i>E. ictaluri</i> (MM19)	1	11	1	9.1	19.1 \pm 6.05 ^{BC}	78.3
	2	10	3	30.0		
	3	11	2	18.2		
pBK	1	11	1	9.1	9.1 \pm 0.00 ^{BC}	89.7
	2	11	1	9.1		
	3	11	1	9.1		
1.1	1	8	2	25.0	8.3 \pm 8.33 ^{BC}	90.5
	2	9	0	0		
	3	9	0	0		
4d6	1	11	4	36.4	27.3 \pm 5.24 ^{BC}	69.0
	2	11	3	27.3		
	3	11	2	18.2		
5d2	1	10	3	30.0	32.7 \pm 9.29 ^B	62.8
	2	10	5	60.0		
	3	11	2	18.2		
5d3	1	9	1	11.1	25.9 \pm 7.40 ^{BC}	70.5
	2	9	3	33.3		
	3	9	3	33.3		
5d4	1	8	1	12.5	23.3 \pm 5.43 ^{BC}	73.5
	2	11	3	27.3		
	3	10	11	30.0		
6d5	1	11	1	20.0	12.7 \pm 3.63 ^{BC}	85.5
	2	11	1	9.1		
	3	11	1	9.1		
Combined	1	11	1	9.1	24.2 \pm 15.15 ^{BC}	72.4
	2	11	1	9.1		
	3	11	6	54.5		

¹RPS = Relative percent survival = 100 x [1 - (% mortality vaccinated + %mortality controls)]

suggests a mortality level below 60% in the challenged non-vaccinated control is unsatisfactory and the test should be considered a "no" test and repeated. Based on this, VT1 was considered to be a failed trial and the results will not be considered further.

To ensure an adequate challenge in VT2, *E. ictaluri* 93-146 underwent two passages through catfish to confirm virulence. Preliminary challenges also showed larger catfish required exposure to a higher concentration of *E. ictaluri* cells in order produce acute infections. Experimental infection procedures were modified accordingly with resulting in a successful challenge in VT2, with 87.9% mortality in the unvaccinated control treatment.

Results of VT2 showed that, compared to the unvaccinated control, percent mortality was significantly lower in the unchallenged control and the vaccinated treatment groups. Although there appeared to be differences in level of protection in different vaccine treatments based on RPS values, due to the high variation between replicates in each treatment and the low number of replicates per treatment and fish per replicate, differences in percent mortalities were not statistically significant. Replicates were limited to three tanks per treatment due to logistical constraints of the high number of treatments (13). The large size of the fish, which averaged 31.5 g, limited the number of fish that could be put in one tank.

Results indicated levels of protection were higher for *E. ictaluri* (MM19) than for *E. ictaluri* (BHI), which had RPS values of 78.3 and 57.9 respectively, though no statistical difference was detected due to variation between replicate tanks. Surprisingly, one of the highest RPS values was seen with the pBK WCL vaccine treatment. This treatment, containing the pBK-CMV expression vector without an *E. ictaluri* insert in the *E. coli* host strain XL0LR, served as the control for the vaccine treatments containing cloned antigenic *E. ictaluri* proteins.

Although the high level of protection of the *E. coli* host strain was unexpected, it was not unprecedented. All bacterial species possess unique proteins, however a surprisingly high number of proteins are shared among various bacterial species (Kaufmann 1993). Catfish *E. ictaluri* antibodies had been previously shown to have a high amount of cross-reactivity with a rough mutant of *E. coli* 0111:B4 (Tyler and Klesius 1994c). The pBK WCL was shown to share

a very high number of cross-reactive proteins with *E. ictaluri* in two-dimensional polyacrylamide gel electrophoresis western blots probed with sera from catfish that had survived *E. ictaluri* infections (Chapter 3). In addition, cross-reactive antigens of *E. coli* were shown to be protective in yearling channel catfish challenged with *E. ictaluri* following vaccination with formalin-killed whole cells of a rough mutant strain of *E. coli* strain (J5) administered once with alum by IP injection (Tyler and Klesius 1994b).

Assessments of protective capabilities of cloned antigenic *E. ictaluri* proteins were inconclusive. Since protective capabilities of the *E. coli* host strain of the cloning vector were so high, protection of the clones could not be ascertained. Additionally, subsequent to the vaccine trials, it was determined through DNA sequencing and protein expression analysis that each of the clones had *E. ictaluri* inserts large enough to encode several proteins, and none of the cloned antigenic proteins were under control of the IPTG-inducible promoter of pBK-CMV vector (Chapter 3). This meant all of the proteins were being expressed based on recognition of cloned *E. ictaluri* transcriptional promoters by *E. coli* RNA polymerase. Because transcription of the cloned proteins was regulated by *E. ictaluri* promoters rather than the strong, inducible vector promoter, the concentrations of expressed cloned proteins were lower than what would be expected for an expression vector. This probably resulted in clones having amounts of expressed cloned proteins that were too low for the WCLs to serve as subunit vaccines.

To correct this problem, genes encoded by *E. ictaluri* inserts will need to be subcloned in the same reading frame as the vector's inducible promoter. Over expression of the cloned proteins may allow differences in protective capabilities of the proteins to be measured compared to the pBK WCL vaccine control. Alternatively, since the vector pBK-CMV is also designed to express cloned inserts under control of the eukaryotic CMV promoter, protective capabilities of cloned antigenic *E. ictaluri* proteins could be assessed using DNA vaccination.

Additional studies using more replicates and perhaps higher numbers of fish per replicate will be needed to determine with certainty if there are any differences in levels of protection between *E. ictaluri* (MM19) and *E. ictaluri* (BHI) WCL vaccines. Subcloning will be necessary to

evaluate protective capabilities of cloned antigenic *E. ictaluri* proteins, and subunit vaccines may not be usable due to the level of protection elicited by the *E. coli* host strain of the cloning vector. Cross-reactive antigens are often conserved T cell antigens (Kaufmann 1993). Although the high RPS of the control pBK WCL inhibited assessment of the cloned *E. ictaluri* proteins, protective capabilities of the *E. coli* due to cross-reactive antigens suggests cross-reactive vaccination may be an additional vaccine strategy to investigate in terms of developing a successful ESC vaccine.

SUMMARY

In this study 2D-PAGE and western blotting with CCS were successfully used to further clarify the identity of antigenic proteins expressed by *E. ictaluri*. This study represents the first reported use of 2D-PAGE and ECL western blotting for the analysis of *E. ictaluri* proteins. It was also the first study to investigate differences in antigenic protein expression of *E. ictaluri* under different conditions of growth. Results showed 2D-PAGE could be used to separate antigenic and non-antigenic proteins of the same molecular weights, and these proteins would appear as one antigenic band in SDS-PAGE western blots. Results also showed differences in strains or routine culture temperatures do not effect expression of antigenic *E. ictaluri* proteins, but culture media does. A unique 57 KD antigenic protein was only expressed by *E. ictaluri* grown in the defined minimal MM19 media and two antigenic proteins of 71 and 58 KD had different levels of expression when *E. ictaluri* was grown in MM19 or enriched BHI media.

In a separate study, a pool of cloned antigenic *E. ictaluri* proteins were generated by screening an *E. ictaluri* genomic library in an *E. coli* expression vector with antiserum against *E. ictaluri*. Nine clones were selected and partially characterized by DNA sequence analysis and protein expression analysis using 2D-PAGE and ECL western blotting with GAI and CCS. Three clones, 4d6, 5d2 and 5d3, were double-strand sequenced and their encoded genes and expressed cloned antigenic *E. ictaluri* proteins were putatively identified. Four putative ORFs were identified in the insert of clone 4d6 corresponding to expressed cloned antigenic proteins of 63, 20 and 18 KD expressed by 4d6 and antigenic proteins of the same molecular weight expressed by *E. ictaluri*. Genes encoding these proteins had no homology with other known genes. Overlapping inserts of clones 5d2 and 5d3 encoded 6362 bp insert with high homology to a region of the *E. coli* chromosome. The insert included partial gene homologs of *E. coli* genes *serA* and *pgk* and complete gene homologs of *E. coli* genes *rpiA*, *iciA*, *yggE*, *yggB* and *fda*. Cloned antigenic proteins of *E. ictaluri* expressed by clones 5d2 and 5d3 were putatively identified. A 33 KD cloned antigenic *E. ictaluri* protein was putatively identified as YGGE, an integral membrane protein with an unknown function encoded by *yggE*. A 27 KD cloned

antigenic *E. ictaluri* protein was putatively identified as ribose 5-phosphate isomerase A, encoded by *rpiA*. A 35 KD cloned antigenic *E. ictaluri* protein was putatively identified as the inhibitor of chromosome initiation of replication A protein, encoded by *iciA*. Finally, a 45 KD cloned antigenic protein of *E. ictaluri* was putatively identified as fructose 1,6-bisphosphate aldolase, encoded by *fda*. Several of these proteins, in particular aldolase, are highly conserved between species. Results of western blot analysis also showed there was a high amount of antibody cross-reactivity between *E. ictaluri* and *E. coli* proteins. Since highly conserved, cross-reactive proteins are frequently T cell antigens, this leaves open the possibility that several of the cloned proteins may be possible T cell antigens. The protective capabilities of 14 cloned antigenic proteins were evaluated in catfish vaccine trials measuring protection from challenge with *E. ictaluri*. All of the clone vaccine treatments had mortality levels significantly lower than the unvaccinated control, however the results were inconclusive due the high level of cross-reactive protection of the *E. coli* host strain of the cloning vector.

This research represents the first study to putatively identify antigenic *E. ictaluri* proteins by more than their molecular weights or locations in the cell. Subcloning is required for definitive identification of the expressed cloned antigenic proteins. Subcloning will also result in fusion proteins with high levels of expression due to the IPTG-inducible pBK-CMV promoter. Due to the higher levels of cloned protein expression, these subclones will probably make better subunit vaccines than the original clones that were tested. If the cross-reactive protection of the *E. coli* can not be overcome by higher protein expression, the plasmid of the cloning vector has a eukaryotic promoter and can be used as a DNA vaccine. This should allow the protective capabilities of the cloned antigenic antigens to be evaluated. Additionally, the high cross-reactive protection of *E. coli* suggests that cross-reactive vaccination may be another vaccine strategy to investigate in terms of developing a successful ESC vaccine.

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VITA

Michelle Moore was born to David Moore and Carol McInerny in Portland, Oregon. Michelle graduated from Milwaukie High School in 1982, and from Oregon State University in 1986. After completing her bachelor of science degree, she moved to Newport, Oregon, and worked as a Port Sampler for the summer with the Oregon Department of Fish and Wildlife, then as an Aquatic Biologist for a year and a half at an environmental consulting firm. While with the consulting firm, she worked for eight months in an on-site effluent characterization study of a oil refinery in the California San Francisco Bay area. After completion of this study, she returned to Newport where she began working towards her master of science degree in Fisheries Science at the O.S.U. Hatfield Marine Science Center (HMSC). While working towards her master's degree, she held an assistantship as an Aquarist at the HMSC public aquarium, where she was involved in the collection and maintenance of temperate marine fishes.

Upon completion of her master's degree in 1991, she moved to Lewes, Delaware, where she worked as a Laboratory Technician spawning and rearing coot clams for genetic studies, and supervising the marine algae culture laboratory. During this time she was married to Michael Burger, whom she had met as an undergraduate at O.S.U. In the summer of 1992 Michelle and Mike moved to Baton Rouge, Louisiana, and both began working towards their doctoral degrees. Michelle completed the requirements for the Doctor of Philosophy in Veterinary Medical Sciences degree in December 1998. In January 1998, she began a National Research Council Post-Doctoral Research Associateship with the National Marine Fisheries Service at the Northwest Fisheries Science Center in Seattle, Washington.

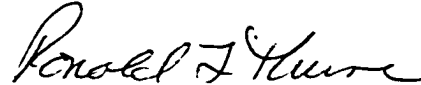
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Candidate: Michelle Marie Moore

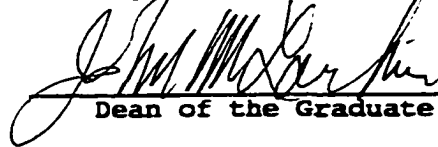
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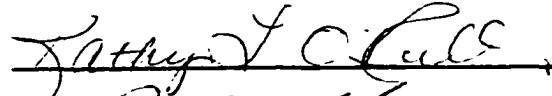


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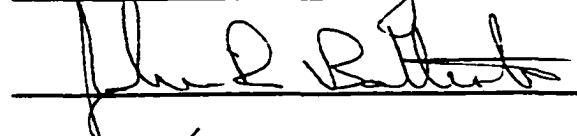
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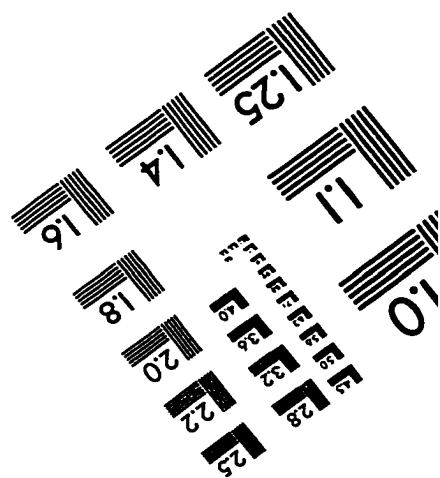
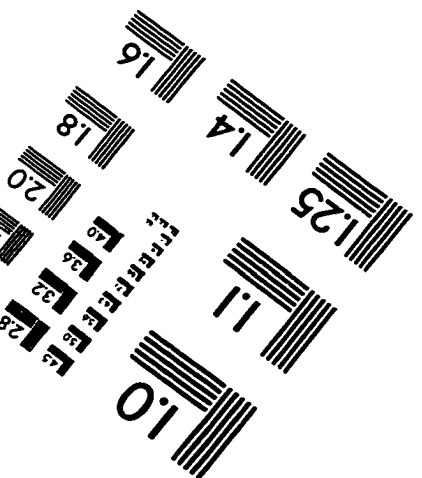
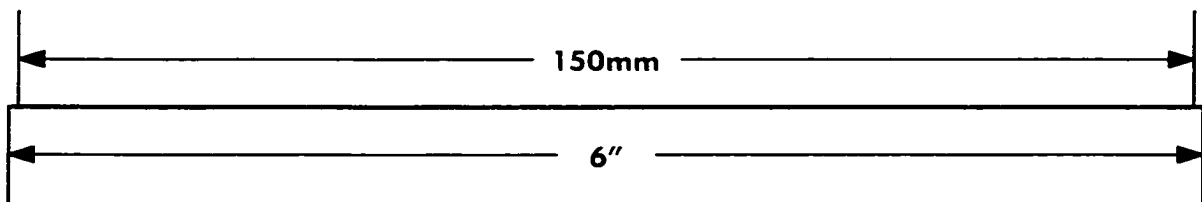
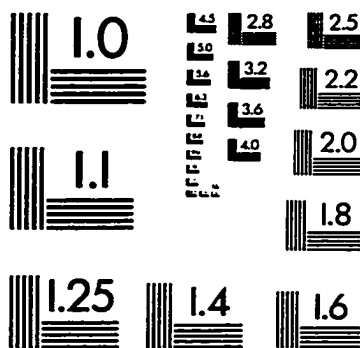
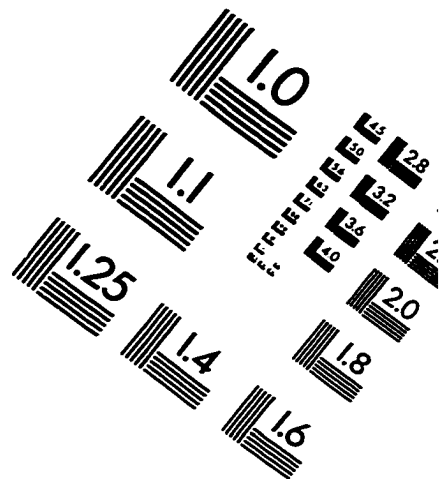
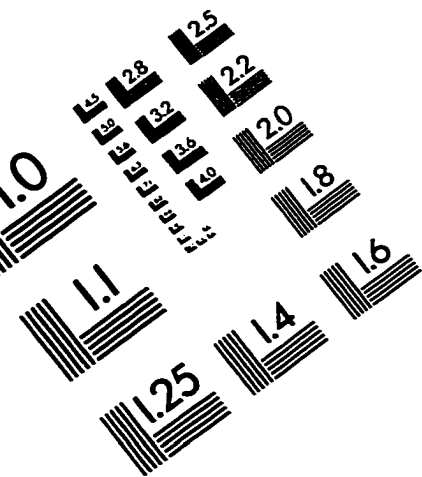


Richard K. Cooper

Date of Examination:

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